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HUMAN HERPESVIRUS 6A AND 6B: ASSAY VALIDATION, VIRUS-HOST INTERACTION AND CLINICAL RELEVANCE

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Cover photo depicting HHV-6B infected SupT1 cells, by Elin Engdahl.
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Human herpesvirus 6A and 6B: Assay validation, virus-host interaction and clinical relevance

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ABSTRACT

Human herpesvirus (HHV)-6A and HHV-6B are two ubiquitous herpesviruses that after primary infection can stay latent in the host. The two viruses can reactivate from latency and cause secondary complications. Also, diseases like multiple sclerosis and epilepsy have been associated with HHV-6A and HHV-6B. However, these associations need to be further examined in order to prove relation and to present evidence for how virus-host interactions can play a role in disease pathology. In order to investigate the effect of viruses in disease it can, at least in some stages, be necessary to perform *in vitro* experiments. When conducting *in vitro* experiments, optimal conditions are needed in order to obtain accurate and reliable results.

The first part of the thesis focuses on improvement of *in vitro* experiential setups. In **Study I**, we sought to develop a correct and robust measurement of virus titers aiming for increased accuracy of experiments and better harmonization within the HHV-6A/6B research field. This was done by optimizing the classical $TCID_{50}$ method with a new qPCR readout. This qPCR readout was found to be more robust compared to other readouts and this method can be used for HHV-6A titer determination. In order to analyze relative gene expression during virus infections, a gene with stable expression is needed for normalization. In **Study II** we searched for a gene with stable expression during HHV-6B infection of Molt-3 cells. Comparison of eight different commonly used reference genes revealed that PPIA is a gene suitable to use as reference gene when working with HHV-6B.

The second part of the thesis focuses on how HHV-6B affects host cell DNA methylation and if this could be one mechanism by which this virus has been associated to epilepsy. **Study III** reveals, for the first time, that HHV-6B induces locus specific host cell DNA hypomethylation close to the telomeres. This hypomethylation was observed already 2 days after infection and correlated with increased gene expression and integration of the virus genome into the host cell genome. No difference in methylation could be observed in epileptic brain tissue which could be due to a transient hypomethylation present only in the acute phase of infection or because of under-powered study design.

The third and last part of the thesis investigates the association between IgG antibody responses against HHV-6A and HHV-6B in MS in order to elucidate if these viruses may play a role in this disease. In addition, the influence of environmental factors and host genetics was investigated. These studies revealed that there was no difference in response against HHV-6B viral lysate between MS cases and controls (**Study IV)** but that established MS patients and pre-symptomatic MS patients had an increased response against the HHV-6A protein IE1A and, to a lesser extent, the HHV-6B protein 101K (**Study V**). The epitope specific IgG responses were highly dependent on the host HLA status. Antibody responses can be interpreted in different ways, but the results of Study V indicate that HHV-6A and HHV-6B may play a role in MS disease etiology. This virus-host interaction in relation to MS needs further investigation.

LIST OF SCIENTIFIC PAPERS

I. **Development and validation of a Q-PCR based TCID50 method for human herpesvirus 6**
Rasmus Gustafsson, Elin Engdahl, Anna Fogdell-Hahn.
Virol J. 2012;9:311

II. **Investigation of reference gene expression during human herpesvirus 6B infection indicates peptidylprolyl isomerase A as a stable reference gene and TATA box binding protein as a gene up-regulated by this virus**
Elin Engdahl, Nicky Dunn, Anna Fogdell-Hahn.
J Virol Methods. 2016;227:47-9

III. **Human herpesvirus 6B induces hypomethylation on chromosome 17p13.3 correlating with increased gene expression and virus integration**
Elin Engdahl, Nicky Dunn, Pitt Niehusmann, Sarah Wideman, Peter Wipfler, Albert J Becker, Tomas J Ekström, Malin Almgren, Anna Fogdell-Hahn.
Manuscript

IV. **HLA-A*02, gender and tobacco smoking, but not multiple sclerosis, affects the IgG antibody response against human herpesvirus 6**
Elin Engdahl, Rasmus Gustafsson, Ryan Ramanujam, Emilie Sundqvist, Tomas Olsson, Jan Hillert, Lars Alfredsson, Ingrid Kockum, Anna Fogdell-Hahn.
Hum Immunol. 2014;75(6):524-30

V. **Serological response against an immediate-early 1 protein sequence from HHV-6A is positively associated with risk for multiple sclerosis**
Elin Engdahl*, Rasmus Gustafsson*, Jesse Huang*, Martin Biström, Izaura Lima Bomfim, Mohsen Khademi, Angelika Michel, Julia Butt, Nicole Brenner, Ingileif Jonsdottir, Kari Stefansson, Daniel Jons, Maria Hortlund, Lucia Alonso-Magdalena, Louis Flamand, Oluf Andersen, Jan Hillert, Lars Alfredsson, Tim Waterboer, Peter Sundström#, Tomas Olsson#, Ingrid Kockum#, Anna Fogdell-Hahn#
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# P.S., T.O., I.K., A.F-H contributed equally to this work and share senior authorship.
Manuscript

ADDITIONAL PUBLICATIONS

I. **Human herpesvirus 6A partially suppresses functional properties of DC without viral replication**
Rasmus Gustafsson, Elin Engdahl, Oscar Hammarfjord, Sanjaya Adikari, Magda Lourda, Jonas Klingström, Mattias Svensson, Anna Fogdell-Hahn.
PLoS One. 2013;8(3):e58122

II. **Anti-interferon beta antibody titers strongly correlate between two bioassays and in vivo biomarker expression, and indicates that a titer of 150 TRU/mL is a biologically functional cut-point**
Christina Hermanrud, Malin Lundkvist Ryner, Elin Engdahl, Anna Fogdell-Hahn.
J Interferon Cytokine Res. 2014;34(7):498-504

III. **First therapeutic use of Artesunate in treatment of human herpesvirus 6B myocarditis in a child**
Nina Hakacova, Karin Klingel, Reinhard Kandolf, Elin Engdahl, Anna Fogdell-Hahn, Thomas Higgins.
J Clin Virol. 2013;57(2):157-60

IV. **Characterization of anti-natalizumab antibodies in multiple sclerosis patients**
Malin Lundkvist*, Elin Engdahl*, Carolina Holmén, Robert Movérare, Tomas Olsson, Jan Hillert, Anna Fogdell-Hahn.
* M.L. and E.E. contributed equally to this work and share first authorship.
Mult Scler. 2013;19(6):757-64

V. **Fatal neuroinflammation in a case of multiple sclerosis with anti-natalizumab antibodies**
Anders Svenningsson, Anna M Dring, Anna Fogdell-Hahn, Iwan Jones, Elin Engdahl, Malin Lundkvist, Thomas Brännström, Jonathan D Gilthorpe.
Neurology. 2013;80(10):965-7.

VI. **Prevalence of anti-drug antibodies against interferon beta has decreased since routine analysis of neutralizing antibodies became clinical practice**
Roger Jungedal, Malin Lundkvist, Elin Engdahl, Ryan Ramanujam, Helga Westerlind, Ajith Sominanda, Jan Hillert, Anna Fogdell-Hahn.
Mult Scler. 2012;18(12):1775-81

CONTENTS

LIST OF ABBREVIATIONS

5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ACTB	β-actin
APC	antigen presenting cell
BCR	B cell receptor
CI	Confidence interval
CNS	central nervous system
CTL	cytotoxic T lymphocyte
CPE	cytopathic effect
CV	coefficient of variation
DC	dendritic cell
DNMT	DNA methyltransferase
dpi	days post infection
DR	direct repeats
EBNA-1	EBV nuclear antigen 1
EBV	Epstein-Barr virus
FACS	flow cytometry
FBS	fetal bovine serum
FDR	false discovery rate
FFWO	fusion from without
GST	glutathione s-transferase
GWAS	genome wide association study
HBLV	human B-lymphotropic virus
HCMV	human cytomegalovirus
HHV-6	human herpesvirus 6
HHV-6A	human herpesvirus 6A
HHV6A/6B	HHV-6A and/or HHV-6B, viral species not determined
HHV-6B	human herpesvirus 6B
HLA	human leukocyte antigen
ici	inherited chromosomally integrated

IE1	Immediate-early 1 protein
IFA	immunofluorescent assay
Inf U	Infectious Units
LD	linkage disequilibrium
LILRB2	leukocyte immunoglobulin like receptor B2
MAF	minor allele frequency
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MOI	multiplicity of infections
MS	multiple sclerosis
MTLE	mesial temporal lobe epilepsy
OD	optical density
OR	odds ratio
ORF	open reading frames
PEST	penicillin and streptomycin mixture
PPIA	peptidylprolyl isomerase A
PPMS	primary progressive MS
qPCR	real time quantitative PCR
RPL13	ribosomal protein L13
RRMS	relapsing remitting MS
SD	standard deviation
SNP	single nucleotide polymorphism
SPMS	secondary progressive MS
TBP	TATA-box binding protein
$TCID_{50}$	50% Tissue culture Infective Dose
TCR	T cell receptor
TET	ten-eleven translocation
Th2	T helper cell type 2
TRS	telomeric repeat sequences
TSA	Trichostatin A

1 INTRODUCTION

1.1 HUMAN HERPESVIRUS 6A AND 6B

In 1986, a new virus was isolated from patients with lymphoproliferative disorders and AIDS. This new virus was given the name human B-lymphotropic virus (HBLV) [1]. However, based on similarities to several herpesviruses it was soon renamed to human herpesvirus 6 (HHV-6) [2]. In the following years, HHV-6 was isolated by several other research groups and it became gradually apparent that the different isolated HHV-6 strains could be divided into two groups. As the two groups differed in their molecular and biological properties, in 1991 it was proposed that the virus species HHV-6 should be divided into group A and group B [3]. Almost 20 years later, after many more publications highlighting the differences between group A and B, HHV-6 was officially replaced by the two distinct virus species HHV-6A and HHV-6B in the genus *Roseolovirus*, subfamily *Betaherpesvirinae*, family *Herpesviridae* [4] (Figure 1).

Family: *Herpesviridae*
- Subfamily: *Alphaherpesvirinae*
  - Genus: *Simplexvirus*
    - Species: Human herpesvirus 1; herpes simplex virus type 1 (HSV-1)
    - Species: Human herpesvirus 2; herpes simplex virus type 2 (HSV-2)
  - Genus: *Varicellovirus*
    - Species: Human herpesvirus 3; varizella zoster virus (VZV)
- **Subfamily: *Betaherpesvirinae***
  - **Genus: *Roseolovirus***
    - **Species: Human herpesvirus 6A (HHV-6A)**
    - **Species: Human herpesvirus 6B (HHV-6B)**
    - Species: Human herpesvirus 7 (HHV-7)
  - Genus: *Cytomegalovirus*
    - Species: Human herpesvirus 5; human cytomegalovirus (HCMV)
- Subfamily: *Gammaherpesvirinae*
  - Genus: *Lymphocryptovirus*
    - Species: Human herpesvirus 4; Epstein-Barr virus (EBV)
  - Genus: *Rhadinovirus*
    - Species: Human herpesvirus 8; Kaposi's sarcoma-associated herpesvirus (KSHV)

Figure 1: Classification of the human herpesviruses, adapted from the International Committee on Taxonomy of Viruses (ICTV) homepage: http://www.ictvonline.org

The primary infection caused by HHV-6A is not clear but HHV-6B was identified as the causal agent of exanthema subitum ("tredagarsfeber" in Swedish) already in 1988 [5]. Since then, many studies have investigated the primary infection of HHV-6B and confirmed the association between HHV-6B infection and acute febrile illnesses with symptoms such as high fever, rashes and febrile seizures in children [6-8]. Less is known about the epidemiology of HHV-6A. Most individuals have mounted an IgG response against HHV-6A/6B (viral species not determined) before the age of two [9-11], indicating childhood infection with HHV-6A and/or HHV-6B in the vast majority of the population.

An additional dimension of herpesvirus infections is that all herpesviruses have the ability to achieve latency in the host [12]. Reactivation of both HHV-6A and HHV-6B might result in potentially serious secondary complications, particularly in immunocompromised patients. For example, HHV-6B reactivation is common in immunocompromised transplant recipients and can lead to encephalitis and other complications in some individuals (reviewed in [13]).

1.1.1 Basic biology

1.1.1.1 Cell tropism

Viruses can enter a target cell by binding of viral glycoproteins to a cellular receptor, after which receptor-mediated endocytosis occurs [14]. One receptor for HHV-6A and HHV-6B is CD46 [15], a cell surface complement inhibitory protein expressed on all nucleated cells. However, it has been discussed that the two viruses interact with CD46 differently [16-18]. A more recently discovered receptor is CD134 (also called OX40), a receptor that specifically interacts with a HHV-6B glycoprotein complex [19]. OX40 is a member of the TNF receptor superfamily expressed on activated T cells that upon binding to its ligand OX40L induce co-stimulatory effects in the T cells [20]. The time between receptor-mediated virus entry into umbilical cord blood lymphocytes and release of new virions is approximately five days [21].

Even though both HHV-6A and HHV-6B primarily replicate in CD4+ T cells, the two viruses grow optimally in different T cell lines highlighting a difference in their biology [22]. Both HHV-6A and HHV-6B can replicate in the T cell line SupT1, but HHV-6A grow optimally in HSB-2 cells and HHV-6B in Molt-3 and MT4 cells [22]. However, other cell types besides T cells can be infected. The two viruses are considered neurotropic as their DNA can be found in human brain tissue *in vivo* [23-26] and because of their ability to infect neuronal cells *in vitro.* For example, human glial precursor cells can be infected by both viruses [27], while only HHV-6A can establish a productive infection in astrocytes [28, 29] and oligodendrocytes [30].

A hallmark of HHV-6A and HHV-6B infection is their cytopathic effect (CPE) on their host cells. Infected cells become enlarged, which can be visualized in a phase contrast microscope (as in Study I). As seen in Figure 2, the infected cells become vacualized though the mechanism

behind this is not well studied. In addition, both HHV-6A [18, 31] and HHV-6B [31] can induce cell-to-cell fusion, resulting in large multi-nucleated syncytia. This virus-induced cell fusion is called fusion from without (FFWO) and does not require viral protein synthesis but is dependent on viral glycoproteins in the virus envelope and CD46 on the cell surface.

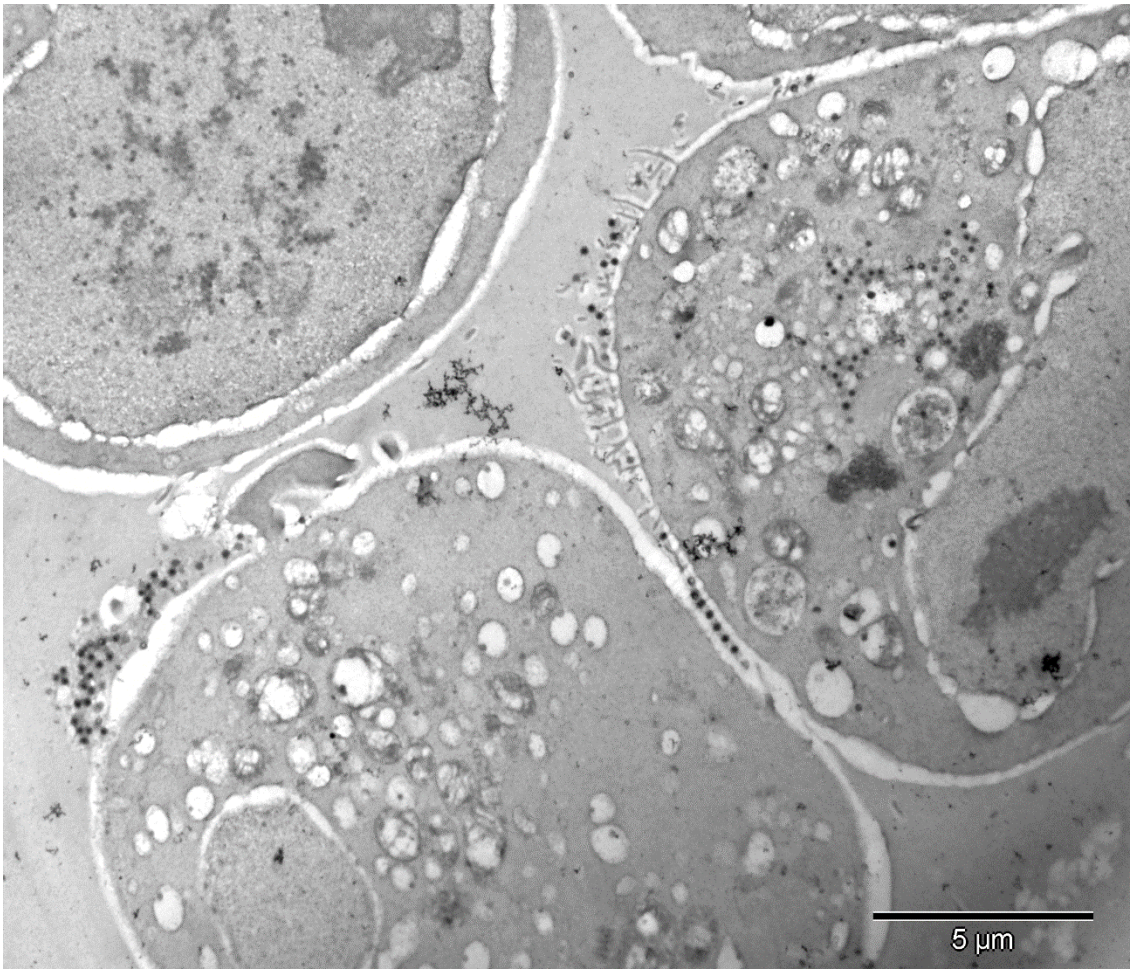


Figure 2: Electron microscopy picture of HHV-6A (GS strain) infected HSB-2 T cells. Both intra- and extracellular viral particles are visible as black round dots. Photo E. Engdahl.

1.1.1.2 Virion structure

Herpesviruses contain large linear double-stranded DNA genomes which are packed within icosahedral capsids. These nucleocapsids (i.e. the genome and capsid) are surrounded by a protein compartment called the tegument. Tegument proteins are essential in several aspects of the viral life cycle, such as for initiation of viral genome replication and modulation of host cell immune responses. A viral envelope, formed of a lipid bilayer membrane containing glycoproteins, surrounds the tegument compartment. This envelope is obtained from the host cell's plasma membrane or cellular organelles during the budding process (herpesvirus structure reviewed in [32, 33], and can be visualized in Figure 3).

Enveloped viruses, like herpesviruses, incorporate not only viral glycoproteins but also host cell proteins and lipids in their envelope [34, 35]. Viral particles will therefore contain the same sort of proteins/lipids that were present in the specific cell type that generated the viral particle. Several different viruses evade complement lysis by incorporating host complement regulatory proteins like CD46, CD55 and CD59 into their envelope [36-38]. Hammarstedt et al. [39] detected CD46 in HHV-6A virions and we have visualized CD46 with immunogold staining on virus envelopes (Figure 3).

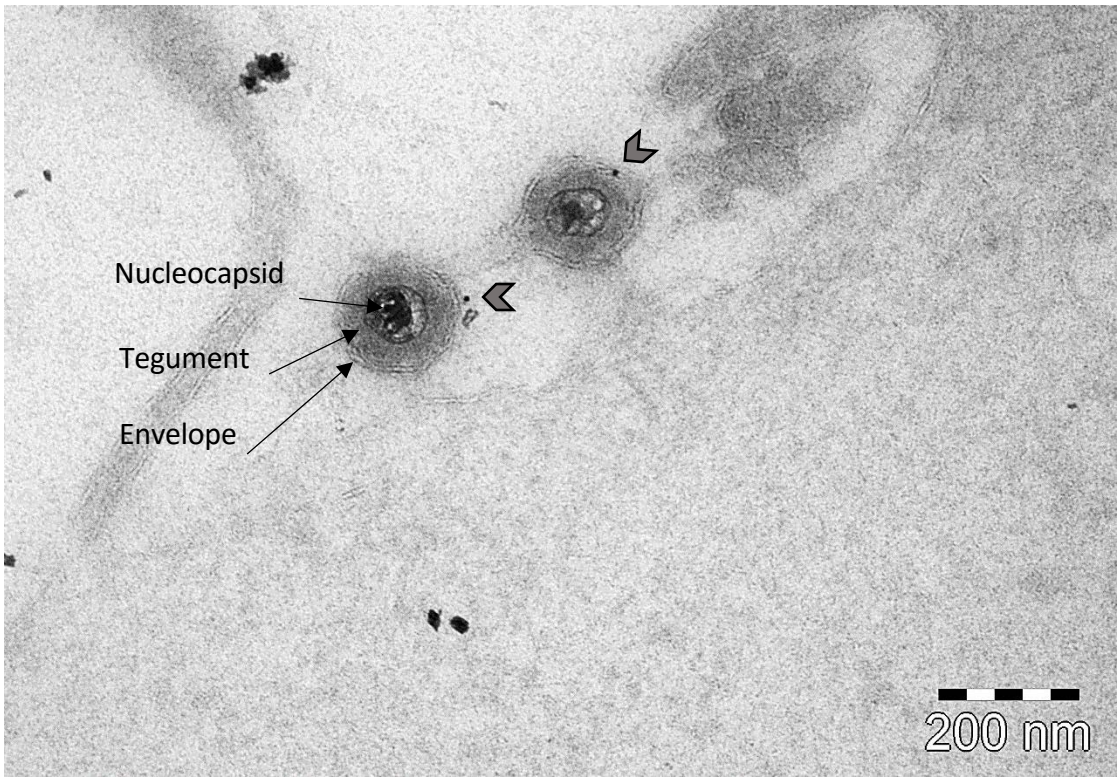


Figure 3: HHV-6A extracellular virions. The viral particles (HHV-6A GS strain) are on the border of an HSB-2 cell, being released to the extracellular space. The black dots are immunogold staining of CD46 (grey arrow head). Nucleocapsid, tegument and envelope indicated with arrows. Photo E. Engdahl.

1.1.1.3 Genes

HHV-6A and HHV-6B share approximately 90% of their overall nucleotide sequence [40, 41] and both consist of a long unique region (U) flanked by left and right direct repeats (DR) (Figure 4). The DR segments contain open reading frames (ORFs, i.e. a part of the genetic code which has the potential to be transcribed and translated into a protein) and two distinct arrays of telomeric repeat sequences (TRS). Each DR segment contains two different TRS regions. At the right end of the DR segments are the "perfect TRS" composed of tandem repeats of the hexamere TAACCC, and at the left end of the DR is the "imperfect TRS" composed of the same TAACCC hexameres but interrupted by other hexamers [41-43]. The number of TAACCC repeats is highly variable between, but stable within, clinical isolates

and different laboratory strains [44]. As the name TRS implies, the complementary hexamere is present in arrays in human telomeric DNA.

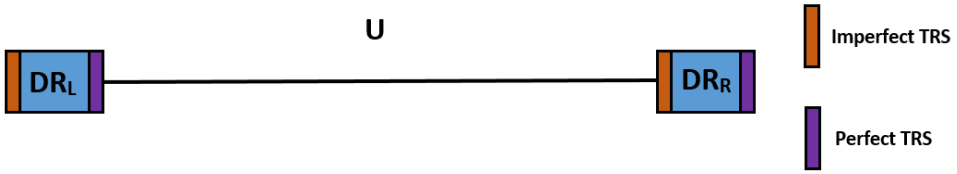


Figure 4. Schematic overview of the HHV-6A or HHV-6B genome. Perfect TRS = a perfect tandem array of human telomeric repeat sequences (TRS), Imperfect TRS = An array of TRS but not in perfect tandem array, U = long unique region, DR_L = Direct repeats at the left side of U, DR_R = Direct repeats at the right side of U. Note that the picture is not in correct scale.

The vast majority of genes are located in the U region. Similar to all herpesviruses, gene transcription follows a temporal regulation with immediate early (IE), early (E) and late genes (L) being expressed [45, 46]. While some parts of the genome are highly conserved between all herpesviruses, other parts are much more species-specific [40, 41].

The HHV-6A and HHV-6B genome contains 39 genes conserved among all mammalian herpesviruses, divided in seven "Herpesvirus core gene blocks", encoding for example structural proteins and enzymes required for DNA replication. One core gene is U66, coding for a protein involved in DNA packaging. This spliced protein has a 99.5% amino acid identity between HHV-6A and HHV-6B [41].

The most divergent part of the genome is the IE-A locus [40, 41]. One protein coded in this locus, in the ORF U90-U89, is the immediate-early 1 (IE1). As with all IE genes, transcription of IE1 occurs within a few hours after virus entry into the cell, and the transcription is independent of prior protein synthesis [45, 47]. The IE1 protein has a 62% amino acid identity between the HHV-6A (IE1A) and HHV-6B (IE1B) [47, 48] and is therefore one of the most divergent proteins between the viruses. Not surprisingly, the proteins not only differ in protein sequence but also in protein function. For example, IE1A is a stronger transactivator of heterologous promoters compared to IE1B [47, 49], indicating a difference in the two proteins' ability to regulate transcription of host cell genes. On the other hand, IE1B plays a role in silencing of IFN-stimulated genes making HHV-6B, but not HHV-6A, resistant to the antiviral actions of IFN-α/β [50]. Another gene with high divergence between HHV-6A and HHV-6B is the late gene U11 that codes for p100 in HHV-6A and 101K in HHV-6B, two tegument proteins with 81% amino acid identity [51]. These structural proteins are essential for virus growth and propagation [52] and 101K has been demonstrated to be a highly immunoreactive protein that is the dominant antigen recognized by HHV-6A/6B IgG [53].

1.1.2 Prevalence studies

1.1.2.1 DNA studies

To investigate the prevalence of HHV-6A and HHV-6B one requires the ability to distinguish between the two viruses. This can at the moment only be done with PCR performed with primers specific for either species. Unfortunately, in the past most research groups have used PCR primers that amplify both HHV-6A and HHV-6B DNA (i.e. cannot distinguish the two viruses from each other), and therefore these studies cannot be used for determination of virus-specific prevalence.

One question that arises is where to look for the virus DNA, which of course depends on the aim of the study performed. If the aim is to investigate the frequency of infected individuals, it is important to analyze tissue where the virus is latent, otherwise infected individuals will be reported as false negative. In addition, the study population should be drawn from the general population.

Most studies that have investigated HHV-6A and HHV-6B prevalence with PCR on DNA from blood and saliva have reported a predominance of HHV-6B [54-57]. For example, HHV-6B prevalence has been reported to be 38-60% in PBMC samples and 67-83% in saliva samples in healthy adults, while HHV-6A DNA was rarely detected in these tissues sites [55-57]. Although blood and saliva are easily accessible, these sites might not be optimal for investigation of HHV-6A prevalence since this virus is rarely detected in these compartments.

Interestingly, the lungs seem to be one important latency site for both HHV-6A and HHV-6B. When looking for HHV-6 DNA in lung tissue of adult immunocompetent control individuals, 100% (14/14) were positive [58]. In a follow up study by the same group, including transplant patients and different controls, they detected HHV-6B DNA in 94% and HHV-6A DNA in 71% of the investigated lung tissue [59]. This indicates that the lung might be a good site for investigation of one individual's infection status, however, it is not a suitable tissue to use for prevalence studies as sampling is problematic. Also, the presence of DNA in tissue exposed to the environment does not necessarily mean infection but could be due to for example degraded virus DNA in immune cells.

1.1.2.2 Antibody studies

Both studies performed by us (Study IV) and others [9, 11, 60-63] have detected IgG antibodies against HHV-6A/6B in >75% of the general population. The problem with antibody assays used, at least until now, is that they have not been able to distinguish between antibodies against HHV-6A and HHV-6B. This makes it hard to use published antibody data in order to study the pathology/biology of the two viral species.

Antibodies, in contrast to DNA, have the advantage that a serum sample can be used to tell if the host has been infected, regardless of infection site in the body. This is of great convenience when investigating HHV-6A as this virus rarely detected in saliva or PBMC. In addition, when investigating the role of viruses in the brain, measurement of antibodies in cerebrospinal fluid (CSF) can reveal intrathecal antibody production allowing an insight to the events inside the blood brain barrier.

There is a need for an assay that can distinguish between antibodies against HHV-6A and HHV-6B in order to study virus specific disease associations. There have been attempts to address this need of a new method. Higashimoto et al. [55] developed an immunoblotting assay with the aim to discriminate between antibodies against 101K, encoded by the HHV-6B gene U11, and p100 by the corresponding gene in HHV-6A. However, this method could not assure absence of cross-reactivity against the proteins. Another group used *in vitro* HHV-6A or HHV-6B infected cells measuring all bound human antibodies with immunofluorescent assay (IFA) [64]. This cannot be regarded as a strict virus specific measurement as many of the expressed proteins are similar between the two virus species. As both the immunoblot assay and the IFA assay are laborious and cannot assure species specificity, a high throughput method with the ability to distinguish between the serological response against HHV-6A and HHV-6B still needs to be developed. This was the aim of Study V.

1.1.3 Latency and integration

Even though the different members of the *Herpesviridae* family have different cell tropisms, mechanisms and associated pathologies, all herpesviruses have two distinct stages in their life cycle: lytic replication and latency. The mechanism through which latency is achieved varies with the different viruses. Both HHV-6A and HHV-6B can achieve latency by integrating its genome into the telomeric regions of host cell chromosomes [65, 66], already during lytic infection [65]. The mechanism behind this integration into host cell genome is not fully understood. However, there are clues.

As mentioned in section 1.1.1.3, the HHV-6A and HHV-6B genomes contain arrays of telomeric repeats (TMRs) within the DR regions. Deletion of these TMRs severely reduces the integration of HHV-6A into host cell chromosomes. When comparing deletion of either the perfect or imperfect TMR, it was discovered that the perfect TMR is most important for HHV-6A integration [67]. Taken together, this study by Wallaschek and colleagues [67] indicates a central role for the TMRs in HHV-6A integration. As HHV-6A and HHV-6B have similar TMRs, it is highly plausible that this also applies for HHV-6B.

A protein suggested to play an important role in HHV-6A and HHV-6B latency is the U94 [68], a protein expressed during IE conditions [45, 46]. This protein is highly conserved between the two viruses (96.5% nucleotide sequence identity [41]), but with no orthologue in the

other human herpesviruses. Nevertheless, U94 has an overall amino acid identity of 24% with the adeno-associated virus type-2 (AAV-2) Rep68 integrase [69, 70]. Interestingly, U94 possesses all functions required for HHV-6A/6B chromosomal integration: it can bind telomeric DNA sequences, hydrolyze ATP and function as both helicase and exonuclease [70]. Even though the properties of this protein seem to be of importance in the integration process, recent findings revealed that HHV-6A integration takes place even in absence of U94 [71].

Another clue to understanding the integration process is that the histone deacetylase inhibitor Trichostatin A (TSA) could reactivate HHV-6A from latent integration [65], indicating epigenetic control of HHV-6A latency.

The HHV-6A and HHV-6B integration is not random but takes place at specific sites. One such site repeatedly described as a chromosomal integration site for both HHV-6A and HHV-6B is the telomere/subtelomere region at 17p [65, 72-75]. Why this location is more suitable for integration compared to other sites is not known. However, sequencing of HHV-6A integration sites revealed that the integration site at 17p contains TMRs distinct from integration sites at other chromosomes [76], possibly facilitating homologous recombination with virus TMRs.

1.1.3.1 ici-HHV-6.

If virus integration takes place in germ cells, there is a 50% probability that the offspring will have this viral genome integrated in every cell of the body. This condition is called inherited chromosomally integrated HHV-6A/6B (ici-HHV-6A/6B) and has been detected in approximately 1% of the human population (reviewed in [77, 78]). As the viral load in a blood sample will be very high, misdiagnosis of active HHV-6A or 6B infection can be a problem. Other clinical implications are still being explored, but it has been reported that reactivation of these integrated viruses can occur [65, 75] and that virus RNA and proteins are occasionally expressed in PBMC from individuals with ici-HHV-6A and ici-HHV-6B [79]. Also, ici-HHV-6A and ici-HHV-6B are associated to different disease groups [75].

1.2 ELICITING AN ANTI-VIRAL B CELL RESPONSE

B cells are generated in the bone marrow throughout life, each new B cell equipped with a unique B cell receptor (BCR). The BCR is a membrane bound IgM or IgD antibody, and as the B cell only has one specificity the later secreted antibodies will be specific for the same epitope as the BCR. Approximately 50% of the B cells in adults are naive B cells circulating between lymphoid organs. The life span of a naïve B cell is a few weeks. However, if a naïve B cell find an extracellular antigen which it has specificity for, the BCR will bind the antigen

starting a cascade of events in the B cell. Most evident, the BCR-antigen complex is internalized, pre-processed and antigen-derived peptides are presented on the MHC class II molecules. Binding of antigen also activates B cells and they start to migrate to the T cell zone of the lymph node where they can meet T helper cells (Reviewed in [80, 81]; Figure 5).

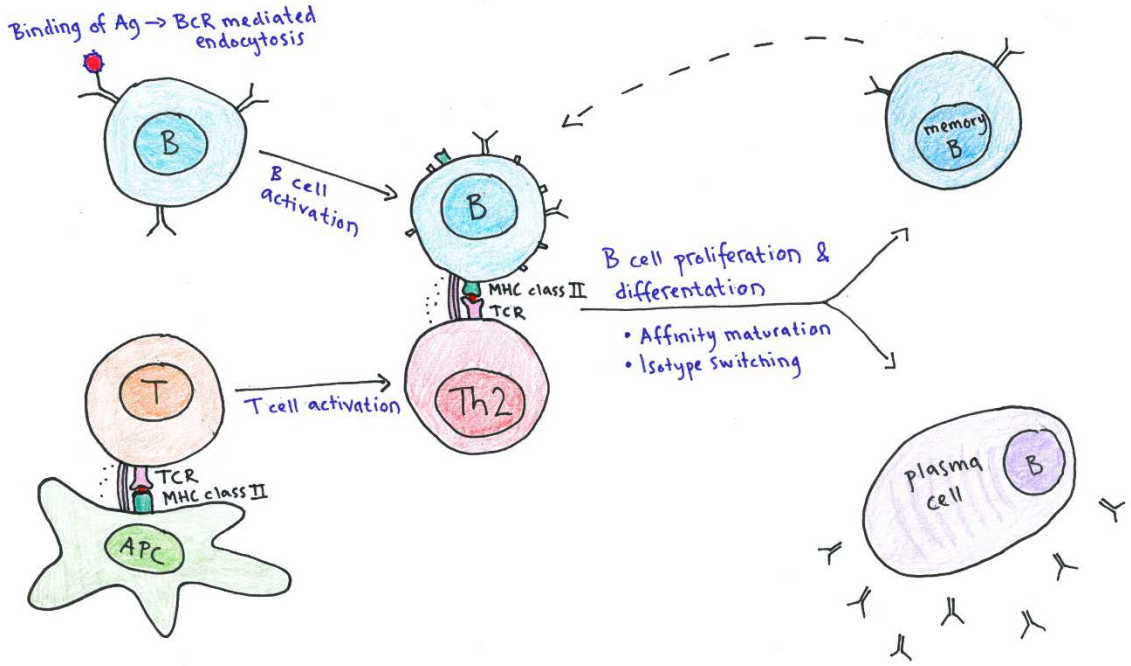


Figure 5: Simplified illustration of B cell activation. When a naïve B cell bind its antigen, it will induce internalization of BCR-Ag complex, B cell activation and presentation of antigen-derived peptides on MHC class II. Increased expression of cytokine receptors also makes the B cell migrate to the T cell zone of the lymph node. There it can meet a Th2 cell already activated by an activated APC. The interaction between MHC class II on the B cell and the TCR on the T cell, with addition of co-stimulatory signals, will induce formation of germinal centers where B cell proliferation starts. Here, the antibody affinity increases through affinity maturation, i.e. somatic mutations in the Ig genes followed by selective survival of B cells producing high affinity antibodies, and the antibody isotype can be changed from e.g. IgM to IgG through isotype switching. B cells differentiate into memory or antibody secreting cells and leave the lymph node. BCR = B cell receptor, Ag = antigen, APC= Antigen presenting cell, i.e. dendritic cell, TCR = T cell receptor, Th2 = CD4+ T helper cell type 2.

In order to become a high affinity antibody-secreting cell, binding between MHC class II on the B cell and the T cell receptor (TCR) on a T helper cell needs to occur. With the addition of co-stimulatory signals and cytokines this binding will induce antibody affinity maturation, isotype switching, B cell proliferation and differentiation into memory B cells and plasma-blasts/plasma cells. Memory B cells can survive for decades and when re-activated they proliferate and either differentiate into plasma cells or re-enter germinal center where they interact with follicular T helper cells and new rounds off affinity maturation and class switching can occur (reviewed in [80]). Plasma cells are terminally differentiated B cells that can produce and secrete large amount of antibodies. There are both short lived and long lived

plasma cells. Long lived plasma cells reside in the bone marrow where they receive growth factors and sufficient support to produce antibodies for years (reviewed in [82, 83]).

1.2.1 Functions of antibodies in anti-viral defense

The first antibody isotype produced is IgM, but as B cells undergo class-switch recombination they can produce IgG and other classes of antibodies as well. There are different effector functions of the different antibody isotypes. The main effector function of IgM antibodies is activation of the complement system. Antibodies of the IgG isotype can be divided in to IgG1, IgG2, IgG3 and IgG4 and they differ in their ability to interact with the complement system and the Fc-receptors. The most common antibody subclass in the human blood is IgG1. One major function of all IgG isotypes in anti-viral response is their ability to neutralize virions, introducing a steric hinder so that the virus particles cannot bind their receptors, hence limiting the spread into other cells. Also, IgG antibodies can opsonize virions and induce Fc-mediated phagocytosis of the viruses. As the IgM isotype, IgG1 and IgG3 antibodies also activate the complement system which can lead to phagocytosis or lysis of viruses and to inflammation (reviewed in [81, 84]). However, viruses have evolved strategies to overcome complement-mediated destruction (see section 1.1.1.2) and thus avoid some of the antibody-mediated anti-viral functions of the immune system.

1.2.2 Role of HLA / MHC in anti-viral defense

As seen in Figure 5, MHC class II peptide presentation is needed in different activation steps in order to elicit an antibody response. First, it is essential in the interaction between antigen presenting cells and naïve CD4+ T cells for their activation into T helper (Th) cells, e.g. with the Th2 profile. Secondly, the MHC class II presentation is needed for Th2 activation of B cells to proliferate and differentiate into memory B cells and antibody producing plasma cells. In addition, MHC class I molecules, expressed on both immune and non-immune cells, constantly present peptides derived from within the cell. If the MHC class I presents for example viral peptides, the infected cells can be recognized and killed by activated CD8+ T cells (i.e. cytotoxic T lymphocytes: CTLs).

In humans, MHC molecules are encoded by human leukocyte antigen (*HLA*) genes. Classical MHC class I molecules are encoded by the HLA-A, -B and –C locus, while MHC class II molecules are encoded by HLA-DR, -DP, and –DQ. Therefore, all individuals will express at least three different MHC class I molecules and at least three different MHC class II molecules on the cell surfaces. The genetic variation within the MHC region is extremely polymorphic: for most of the HLA genes there are very many different alleles, i.e. variants of the genetic locus. All HLA-alleles are nowadays named in a certain, very structured way with two-digit numbers separated by colons [85]. Most individuals are HLA heterozygous (i.e. different

alleles on the two copies of chromosome 6) and as there is expression of both alleles the ability to bind different antigens increases even further. Taken together, the HLA molecules in one individual should be able to present several antigens from a viral pathogen, making it impossible for the infection to go undetected by the immune system. The extensive HLA polymorphism in the population ensures that a virus will be detected differently by different individuals and thus limit the success of virus escape mutants in a population. The association between different HLA alleles and the ability of MS patients and healthy controls to mount IgG responses against different HHV-6A and -6B epitopes was investigated in Study V.

1.3 HHV-6A AND HHV-6B ASSOCIATION TO DISEASES

1.3.1 Multiple sclerosis (MS)

Multiple sclerosis is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS) with autoimmune features. The average age at onset is 30 years and as with many autoimmune diseases, women are overrepresented. The disease is characterized by demyelinated areas in the brain and/or spinal cord. In a healthy brain, neuronal axons are wrapped with myelin sheaths produced and maintained by oligodendrocytes. Compact myelin segments separated by unmyelinated nodes of Ranvier are needed for correct nerve signaling. The demyelinated areas in MS are called plaques or lesions. These lesions are caused by immune cell infiltration into the brain that induces inflammation, demyelination, gliosis and axonal damage (reviewed in [86, 87]).

Multiple sclerosis is a heterogeneous disease with different disease courses, neurological symptoms and progression rates. The most common form of the disease is relapsing-remitting MS (RRMS) affecting approximately 80-85% of patients. During relapse, CNS inflammation and demyelination occur and neurological symptoms arise, but between relapses re-myelination and complete remission can occur. As time goes by, the improvement between relapses disappears and disability accumulates and eventually approximately 80% of RRMS patients convert into secondary progressive MS (SPMS). This phase is less inflammatory but instead axonal loss and decreased brain volume is characteristic and during this phase there is a constant disease progression (reviewed in [86, 87]) (Figure 6).

Despite many years of intense research trying to find a cause to this complex disease, the main conclusion is that the disease is multifactorial and that there is not one specific causative agent. Both genetic and environmental factors are considered to contribute to disease susceptibility. Genetic factors influencing MS susceptibility are primarily immune genes [88] with human leukocyte antigen (HLA)-DRB1*15 being the strongest risk allele [89, 90] and HLA-A*02 being a protective allele [89, 91]. Environmental risk factors include smoking [92], low vitamin D levels (reviewed in [93]) and infections (reviewed in [94]).

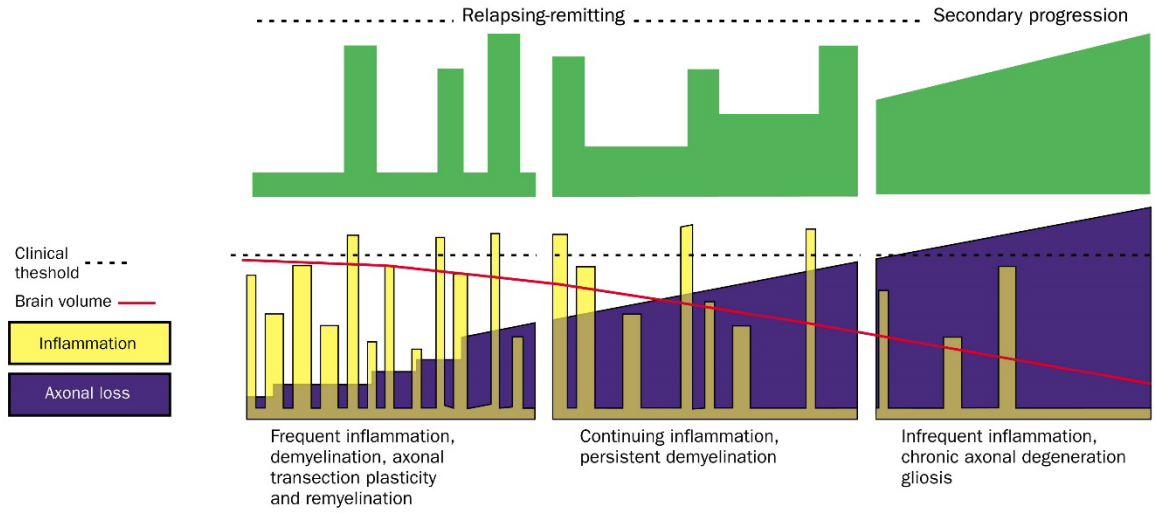


Figure 6: Clinical course of MS. The top row (green) shows the clinical symptom of the disease with a relapsing-remitting onset later converting to a secondary progressive course. In early stages of MS, the inflammation is more profound but as the disease progresses, the brain volume decreases as the axons are destroyed (bottom row). Reprinted from Lancet, 2002, 359(9313):1221-31 [86] with permission from Elsevier.

Epstein-Barr virus is one of the most studied viruses in the MS research field, where both infectious mononucleosis and antibodies against the Epstein-Barr nuclear antigen 1 (EBNA-1) have been associated with MS disease [95-98]. Also HHV-6A [64, 99-101] and HHV-6B [26, 102] have been repeatedly associated with MS. For example, the HHV-6B protein 101K has been observed more frequently in oligodendrocytes in MS lesions compared to in normal appearing white matter from MS patients and controls [26, 102], indicating a role of the virus at disease site. Also, HHV-6A DNA has been observed in CSF of 22% of MS patients but in 0% of controls [100] and intrathecal anti-HHV-6A/6B IgG antibody production has been identified in approximately 25% of MS patients [60, 103]. When studying the role of HHV-6A and HHV-6B during disease, Alvarez-Lafuente et al. [99, 104] have in several studies demonstrated a role of HHV-6A in disease activity as they observed higher HHV-6A viral DNA load in serum, and messenger RNA load in blood, during MS relapse compared to remission. The same group has also recorded an increase in anti-HHV-6A/6B IgM prior to relapse [61]. Since IgM antibodies are regarded as markers of recent reactivation or primary infection, the increased anti-HHV-6A/6B IgM prior to relapse suggests that the observed HHV-6A reactivation during relapses may have had a trigging effect on the relapse onset rather than being a consequence of it. Also suggestive of a role in disease onset, another group observed increased levels of IgM antibodies against HHV-6A/6B in early MS stages [105]. Thus, the association between viral infections and MS is interesting, but it is still unclear how such triggering mechanism would work. Three suggested mechanisms for viral triggering of autoimmunity are molecular mimicry, bystander activation and epitope spreading (reviewed in [106]). Another theory is that virus-incorporation of host cell proteins can induce autoimmunity against the incorporated molecules [107] (more about this hypothesis in section 4.4.6).

Not only is the trigger of MS unclear, but also the pathological processes by which the immune system interacts and degrades the myelin is complex and not fully understood. An extensive focus has been set on understanding the T cell mediated pathogenesis in MS, but the role of B cells and other cell types has received increased attention in the last few years. T cells are located within the MS lesions in early stages of MS disease, and the main CD4+ T cell subsets are T_H1 and T_H17. CD8+ T cells are also found in lesions and their number correlates with axonal damage. There is not only one specificity of these cells but the main specificity is thought to be against different myelin proteins (reviewed in [87]). B cells have received increased attention lately as treatment with B cell depleting anti-CD20 antibodies decreases disease activity [108-110].

1.3.2 Epilepsy

Epilepsy is a disease characterized by uncontrolled over-excitatory activity of neurons, which results in seizures and convulsions. There are different causes for the disease and in many cases the etiology is unknown. Epileptic seizures can be explained by electric discharges of the neurons that cannot be turned off. This could be due to an impairment of the neurons themselves or lack of regulation from the supporting glial cells, e.g. astrocytes. To date, there is no cure for epilepsy, but the seizures can be controlled by drugs. In severe medically refractory epilepsy, surgery is used to remove the part of brain tissue from where the seizures originate.

High HHV-6B viral DNA load has been observed more frequently in surgically removed hippocampus tissue from mesial temporal lobe epilepsy (MTLE) patients than in non-epileptic tissue [111-114], indicating an association between the virus and epilepsy. In the epileptic tissue, HHV-6B has been found active in astrocytes [111, 112]. Astrocytes are the supporting cells of the brain with many functions including provision of nutrients to neurons and clearing of neurotransmitters in the brain so that the signaling between neurons can be terminated. Interestingly, HHV-6B infected astrocytes have dysregulated glutamate uptake [115]. Glutamate is an excitatory neurotransmitter and its extracellular levels need to be tightly regulated [116], hence deficient glutamate clearing by infected astrocytes might be one mechanism that could explain epileptic symptoms. Indeed, epileptogenic hippocampus has abnormally high levels of extracellular glutamate [117], indicating insufficient glutamate clearing in the epileptic tissue.

In recent years, it has been suggested that epigenetic modulations may play a role in epilepsy [118, 119]. For example, the methylating enzymes DNMT1 and DNMT3a are strongly expressed in epileptogenic brain tissue from temporal lobe epilepsy patients, but only weakly expressed in control brains [120]. More methylation would generally indicate less gene expression in epileptic tissue, and indeed, hippocampi of temporal lobe epilepsy patients have

less expression of glutamate transporters [121]. However, none of these studies investigated the presence of viruses in the tissue.

1.4 EPIGENETICS

All cells of one individual carry exactly the same genetic code. However, the phenotype of different cell types varies enormously. This is because "epigenetics", which could be explained as information associated with the DNA, but not encoded by the DNA sequence itself, affects the expression of the genome hence creating the cell phenotype. This information is generally inherited from mother cell to daughter cell so that the cell phenotype remains after cell division. However, the epigenetic code can be altered by environmental factors like stress, infections, diet, smoking and physical activity (reviewed in [122-126]).

The two most studied mechanisms controlling epigenetic changes are DNA methylation and histone modifications. There is an interaction between different epigenetic regulating factors and several epigenetic modifications are needed for a strong effect. There is for example a strong correlation between DNA methylation, histone deacetylation, tightly packed chromatin and transcriptional repression (reviewed in [127]).

1.4.1 DNA methylation

DNA methylation is when a methyl group is covalently added to the carbon-5 position of a cytosine base in the DNA. This cytosine then becomes a 5-methylcytosine (5mC). The methyl transfer is executed by DNA methyltransferases (DNMTs) and primarily occurs at CpG sites, i.e. where the cytosine nucleotide is followed by a guanine nucleotide separated only by a phosphate. There are different DNMTs; DNMT1 is a key player in maintaining methylation marks after genome replication, while DNMT3a and DNMT3b can induce new methylation marks (*de novo* methylation) [128].

Even though methylation is regarded as a relatively stable modification, methylated cytosines can be de-methylated. This can happen by passive de-methylation or by active oxidation executed by ten-eleven translocation (TET) enzymes (Figure 7). Passive de-methylation refers to lack of methyl groups or methyl donners, or absence of DNMTs. In active de-methylation, oxidization of the methyl group of 5mC to hydroxymethyl, formyl or carboxyl groups will convert 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) or 5-carboxy-cytosine (5caC). These oxidized products will then be converted to unmethylated cytosine (reviewed in [129, 130]).

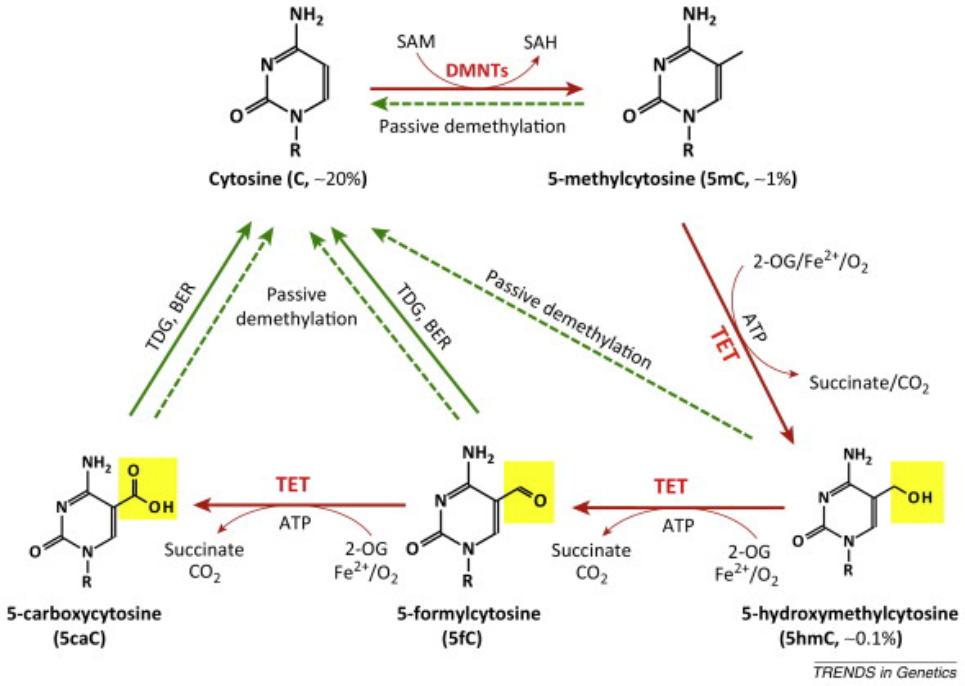


Figure 7: Major DNA methylation and demethylation pathways in mammals. DNMTs = DNA methyl-transferases; TET = ten eleven translocation enzymes; SAM = S-adenosylmethionine; TGD = thymine DNA glycosylase; BER = base excision repair. Reprinted from Trends in Genetics, 2014, 30(10): 464-474 [129] with permission from Elsevier.

1.4.2 DNA methylation during virus infection

The host epigenetic machinery is used in the defense against viruses in order to silence their transcription. It has been suggested that latencies of several herpesviruses are regulated by epigenetic mechanisms [131-133]. Both DNA methylation and histone modifications are involved in this regulation but this section will focus on DNA methylation as this is the focus in Study III. DNA methylation has been observed to regulate expression of many genes in the Epstein-Barr virus (EBV) genome, a way to control latency of this virus [134, 135]. DNA methylation has also been suggested to play a role during human cytomegalovirus (HCMV) latency as inhibition of DNA methylation activates silenced HCMV promoters [136]. In addition, non-susceptible cells became susceptible to HCMV infection upon inhibition of DNA methylation, indicating that the host cell methylation machinery is needed in the anti-HCMV defense during infection [137].

As pathogens usually do, viruses have evolved strategies to overcome the immune regulation by the host, for example to use the epigenetic machinery for their own survival (reviewed in [123, 131, 138]). Esteki-Zadeh et al. [137] observed that HCMV affects the host DNA methylation capacity by inducing re-localization of DNMTs from the nucleus to the cytosol, hindering these enzymes to execute their methylating function. This re-localization induced global hypomethylation in the infected cells (i.e. less methylation compared to control), primarily due to an increase of un-methylated virus DNA. Virus modulation of the epigenetic

machinery can have severe implications for the host cells as many genes can be affected. One example is cancer caused by EBV infection, where EBV induces expression of DNMT3b, resulting in increased DNA methylation and decreased expression of many important host cell genes [139, 140]. To date, there are no publications investigating how HHV-6A and HHV-6B affect host cell DNA methylation. However, as other herpesviruses affect host cell DNA methylation it is plausible that HHV-6A and HHV-6B also has this capacity. The effect of HHV-6B on host cell DNA methylation was investigated in Study III.

1.5 GENETIC ASSOCIATION TO INFECTION

As the virus life cycle is dependent on host cells, the virus - host interaction is of interest. One question to answer is if genetic variations in the host genome can affect this interaction.

The human genome is in general very similar between individuals. However, differences exist for example through insertions/deletions of genomic sequences and through copy number variations (i.e. repeated genomic sequences in different number of repeats). The most studied genetic differences are certain positions in the DNA that consist of different nucleotides in a population. These sites are called single nucleotide polymorphism (SNP) and they can be analyzed and associated to different outcomes, most often diseases.

One approach is to investigate the genetic association between one selected SNP, presumably based on a hypothesis or previous results, and the infection of interest. Using this approach, an association between HHV-6A active replication, defined as serum HHV-6A DNA, and the SNP rs4774C in the *CIITA* gene (class II major histocompatibility complex transactivator, Also known as: MHC2TA) was detected [141]. This gene encodes a protein that acts as a transcriptional regulator of MHC class II gene transcription, indicating MHC presentation to be important in HHV-6A viral control.

1.5.1 Genome Wide Association Studies (GWAS)

Another approach for investigating genetic associations to the virus of interest is to do a hypothesis-free genome wide association study (GWAS). First, genotypes for numerous SNPs (e.g. approximately 600,000 SNPs in Study V) are determined using arrays. When obtaining this kind of huge data sets from the genotyping, the data first needs to be quality controlled. When all SNPs not fulfilling certain criteria have been removed, an association study is made.

As each SNP is investigated for association with the trait, the number of associations calculated are enormous. This needs to be taken into account when calculating the statistics, as accepting a type 1 error of 5% often used in experimental studies would yield an unacceptable number of false positive associations. A standard p-value accepted for

significance in GWA studies is $p<5x10^{-8}$. As the p-values need to be very low for significance, the included patients/controls need to be in great numbers. The power to detect a difference is also dependent on having a clear qualitative trait, e.g. correctly defined seropositive and negative individuals, or a correctly measured quantitative variable, e.g. antibody levels.

SNPs can be in linkage disequilibrium (LD), i.e. in non-random association because of a nearby location in the genome which makes them inherited together more often than expected by chance [142, 143]. Regions in high LD can be called haplotype blocks. This structural architecture can be used when deciphering GWAS data as not all SNPs in the genome have to be genotyped. The awareness of LD is of highest importance when focusing on the MHC region where several different alleles can be located in one haplotype [144]. SNPs annotated to non-HLA genes within the MHC region may be significantly associated to the investigated trait only as a consequence of being in an associated HLA haplotype. Also outside the MHC region, one needs to consider that a non-causing SNP can be significantly associated to the trait of interest only because this SNP is in LD with a causing SNP not investigated.

1.5.1.1 GWA studies and herpesvirus infections

When investigating genetic association with virus infection, it may not be obvious how to define the groups to compare. As mentioned above, Martinez et al [141] used serum HHV-6A DNA as a marker of active HHV-6A infection. It is however hard to find enough patients with this trait to do a full GWAS. As mentioned in section 1.1.2, an accurate latent or active infection status of one individual depends on sampling strategy and might not classify the patients correctly. A more indirect way is to measure antibody response against the virus of interest. However, this induces the uncertainty of what is genetically associated to the virus pathology and what is associated to the ability of the host to mount an antibody response.

Rubicz et al [145] investigated the genetic associations to antibodies against several common viruses, for example against HHV-6B lysate, in a GWAS. They observed no significant association between HHV-6A/6B serostatus and investigated SNPs. Another study by Hammer et al [146] found that the IgG response against the EBV nuclear antigen 1 (EBNA-1), the Influenza A virus and the major capsid protein VP1 from JC virus was associated to SNPs in the MHC region. They could specifically pinpoint these genetic associations to amino acid residues in MHC class II proteins likely causing the association with antiviral antibody response against these viruses. As expected, the associations were virus-specific and not similar between all anti-viral responses.

2 AIMS

The overall aim of this thesis was to increase the knowledge of HHV-6A and HHV-6B in relation to epilepsy and MS.

Specific aims:

Study I and II: To get better control over the experimental settings and increase the accuracy of the results. More specifically, the aim of Study I was to develop a robust and correct titer determining assay and the aim of Study II was to analyze stability of different reference genes in order to determine the most suitable reference gene to use in relative gene expression analyses in HHV-6B experiments.

Study III: To investigate how HHV-6B affects DNA methylation. In addition we aimed to explore if DNA methylation changes induced by HHV-6B can have a pathogenic role in epilepsy or a role in the virus integration process.

Study IV: To investigate the association between anti-HHV-6A/6B IgG response and MS disease.

Study V: To develop a more specific antibody measurement and further investigate the role of HHV-6A and HHV-6B in MS. In addition, we aimed at exploring the genetic associations to the measured anti-viral IgG response.

3 MATERIAL AND METHODS

3.1 STUDY I

3.1.1 Study design

The most basic information required when carrying out experiments with pathogens is how infectious the batch is that you're working with. Culturing of HHV-6A in HSB-2 cells is a skill and we had problems with low infectious virus batches and no suitable method to measure what we were working with. There is no accepted standard measurement of HHV-6A/6B virulence and we were in great need of an easy, robust and correct measurement. So, what were the options?

Plaque forming assays used for other viruses like HCMV [147] are not possible as HHV-6A and HHV-6B do not induce plaque formation. Also the fact that these viruses primarily infect T cells which are in suspension and not adherent induces some limitations.

Another way to measure virus titers is to use the 50% Tissue culture Infective Dose ($TCID_{50}$) endpoint dilution assay [148]. The interpretation of infectivity in this assay can be made with different readouts. The most common way to check for HHV-6A and HHV-6B infected cells is to look for enlarged cells (i.e. CPE) in a microscope. However, this is subjective and can be influenced by what one wants to see. The evaluation of CPE also relies on experience of assessing HHV-6A/6B infection in the cells of choice as even uninfected cells can become a bit "enlarged". Also, there are size differences between the cell lines used for HHV-6A and -6B propagation. We had used ocular inspection when propagating virus batches in order to follow infection, but it did not seem to correlate very well with the infectivity of the virus batch obtained in the end and we did not feel confident to use this readout in a virus titer determining assay. Expression of genes/proteins can be good markers of active infection and possible to use as readout for the $TCID_{50}$ plate. However, investigating virus RNA includes both RNA purification and conversion to cDNA, plus the use of correct normalization. IFA staining of virus proteins includes fixation of cells, staining and manual counting of an enormous amount of cells in the microscope. We decided both these readouts to be too laborious.

There is one method used by some researchers in the field called Infectious Units (Inf U) that is based on IFA staining of cells [149]. This method relies on the assumption that the cells have not changed in numbers during the incubation period, which can be a problem when investigating low-titer batches as the cells might divide during this time. In addition, the interpretation of the fluorescent signal of the cells can be a bit subjective as it is not always clear what is positive and negative in the microscope.

Some laboratories do not use biological assays at all, but instead use the amount of viral DNA copies in the virus batch as a measurement for infectivity. This relies on the assumption that all viral copies are from complete viruses able to infect a host cell, which we did not believe in.

In the end, we chose to optimize the classical $TCID_{50}$ assay and developed a new PCR based readout.

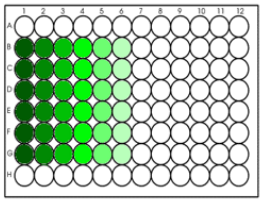
3.1.2 Cell culture and HHV-6A propagation

A vial containing passage 10 of the HHV-6A GS strain, which had been stored since 1987, was kindly provided from Helena Dahl at the Swedish Institute for Infectious Disease Control, and propagated in the T-cell line HSB-2 using GlutaMAX containing RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone), 100 U/ml penicillin and 100 µg/ml streptomycin mixture (PEST) (Invitrogen), referred to hereafter as RPMI complete medium. When approximately 50% of the live cells were enlarged, the culture was spun at 280 x g for 10 minutes and the supernatant collected and frozen immediately in aliquots in -80°C until analysis. Fractions of HHV-6A passage 17 (P17) were inactivated by UV- or heat-treatment for 20 min or 1 h at 56°C respectively.

3.1.3 $TCID_{50}$ method with different readouts

In order to determine the optimal time when to analyze our $TCID_{50}$ assay, a growth curve was made. HSB-2 cells were inoculated with different amount of HHV-6A and samples were collected at different time points. DNA was extracted simultaneously from cell suspension using a commercial 96-well magnetic bead based kit according to the manufacturer's protocol (MagMAX-96 Viral RNA Isolation Kit, Applied Biosystems). DNA load was analyzed by real time quantitative PCR (qPCR) (7500 Fast Real-Time PCR System, Applied Biosystems) using primers for an immediate-early gene previously described [150]. Based on this growth curve, we chose 7 days post infection (dpi) as the end point. $TCID_{50}$ plate setup is visualized and described in Figure 8.

At 7 dpi, the stored 0 dpi culture plate was thawed and cell suspension from every well from the both time points was added onto the same DNA extraction plate enabling the entire extraction procedure to be completed within 1 hour. Thereafter, the viral DNA was quantified by qPCR as described above. Wells where the viral DNA load had increased ten times at 7 dpi as compared to 0 dpi were considered as infected. The $TCID_{50}$ was calculated according to the classical formula by Reed and Muench [148]. The units obtained are in the format of $TCID_{50}$/ml and interpreted as the dilution giving infection in 50% of the wells. For example, a 1215 $TCID_{50}$/ml means that if 1 ml of a 1:1215 dilution of this batch is added to wells (containing cells as in the $TCID_{50}$ plate), 50% of the wells will contain infected cell cultures.



HHV-6A was seeded in 5-fold dilution series (sextuplicate wells). Triplicate wells of mock were included on all plates. 10^4 HSB-2 cells were added to each well. The plate was incubated for 3-4 hours, washed and fresh media was added.

Samples from each well frozen in -80°C

Plate incubated 7 days in 37°C, 5% CO_2

DNA was extracted using MagMax-96 Viral RNA/DNA isolation kit from both the day 0 and day 7 samples. qPCR were run on all wells.

Positive wells = viral DNA increase with at least one Log10 from day 0 to day 7

$TCID_{50}$ values were calculated according to the Reed-Muench method

Figure 8: $TCID_{50}$ plate setup. 160 µl of the examined virus batch was added in sextuplicate wells to the first column of a round bottom 96 well culture plate and titrated in five-fold dilution steps. Then 10^4 HSB-2 cells (40µl) were added to each well and after incubation and washing of the plate, fresh RPMI complete media was added after which cell suspension was sampled from every well and stored in -80°C. The remaining cell suspensions were incubated for 7 days at 37°C and 5% CO_2.

In order to investigate the accuracy of our PCR-based readout, we wanted to compare our results with results obtained with other methods from the same plates, also at 7 dpi. All $TCID_{50}$ plates were assessed by ocular inspection for enlarged cells using a phase contrast microscope by two independent inspectors. Wells where at least one enlarged cell was found were considered as infected. Viral protein expression was investigated with IFA on cells from every well of six $TCID_{50}$ plates. The cells were fixed onto glass slides with a 1:1 mixture of acetone and methanol at -20°C for 10 minutes, thereafter blocked with 5% goat serum in PBS with 3% BSA. Staining was performed using primary mouse monoclonal antibodies specific to the HHV-6A/6B viral glycoproteins gp116/54/64 (1:60, Tebu-bio). The staining was visualized by an Alexa 633 conjugated secondary goat anti-mouse IgG (1:1000, Invitrogen). In addition, cell nuclei were stained with DAPI (Vector laboratories). Wells where at least 2% of the cells contained viral proteins were considered as infected.

In order to compare stability of the methods, coefficient of variation (CV) was calculated (Formula 1). Variation of the readout, i.e. intra-assay CV, was calculated by comparing repeated measurements of one readout for one plate. Variation between plates, i.e. inter-assay variation, was calculated by comparing the results obtained for one virus batch using the same method.

$$Coefficient\ of\ variation\ (CV) = \frac{standard\ deviation}{average}$$

Formula 1: Coefficient of variation (CV). CV is a measurement of variation among a set of values. CV can be given as % CV, i.e. the calculated CV x100.

3.1.4 Other titer determinations

Assessment of Inf U by IFA was performed as previously described [149]. Briefly $2.5x10^5$ un-infected HSB-2 were inoculated as described above with 100 µl of undiluted or 10, 50 or 100 times dilutions of HHV-6A in triplicate wells. The plate was incubated for two days before the cells were fixed onto glass slides and stained for the early viral protein p41 (1:50, Santa Cruz Biotech. Inc.). The viral titer, expressed as infectious units per ml, was calculated by multiplying the fraction of infected cells with the total number of cells at 0 dpi and with the dilution factor.

As a comparison to these titer-determining assays mentioned above, the number of DNA copies were also determined in the virus batch. DNA was extracted from the virus batch using a commercial kit (QIAamp Viral RNA Mini Kit, QIAGEN) according to the manufacturer's instructions. The viral DNA load was quantified with qPCR as described above but with the addition of a standard curve with known number of HHV-6A DNA copies.

3.2 STUDY II-III

3.2.1 *In vitro* experiments (Study II – III)

HHV-6B strain Z29 was propagated in the Molt-3 T-cell line. Virus-containing cell free supernatant was obtained by centrifugation for 15 min at 280 x *g* when the cytopathic effect in the cell culture was >50%, and aliquots were stored at -80°C. Supernatant from uninfected Molt-3 cells was harvested in the same way as for the virus-infected cells and used as mock in subsequent experiments. Virus titers were determined using the qPCR readout of the $TCID_{50}$ method described in Paper I, although changing the HSB-2 cells to Molt-3 cells and the HHV-6A specific primers to HHV-6B specific primers [151].

Molt-3 cells were incubated with mock or HHV-6B at a multiplicity of infections (MOI) of 0.01 and 0.001 for 3h before medium was changed to fresh RPMI medium supplemented with GlutaMAX (Gibco), 1% PEST (Gibco) and 10% FBS (Gibco), and further cultured in the presence of 5% CO_2 at 37°C. Three separate experiments were started at different days, each containing triplicate cultures for all conditions.

All cell cultures were stained for the viral protein p41 (Santa Cruz Biotechnology) at 3 dpi yielding 96% (±1%) and 38% (±7%) positive cells in the HHV-6B cultures (0.01 MOI and 0.001 MOI respectively) while 0% positive cells in mock-treated cells cultures.

3.2.2 *In vivo* material (Study III)

As one of the original aims of the thesis was to investigate the role of HHV-6B in epilepsy, we wanted to investigate differences in epileptic tissue infected with HHV-6B compared to uninfected. To obtain material from epileptic brain surgery performed at Karolinska University hospital was found to be very hard. However, at the 7th international conference on HHV-6 & 7 in 2011 I had the opportunity to meet Dr. Pitt Niehusmann, a neuropathologist working with HHV-6A/6B in epilepsy at University of Bonn Medical Center. A collaboration was initiated and he sent purified DNA and RNA from their MTLE cohort, already investigated for presence of different viruses, including HHV-6B [152]. Twelve patients positive and 12 patients negative for HHV-6B DNA in their surgically removed epileptic brain tissue were selected for inclusion in Study III. For detail see Supplementary table 1 in Study III.

3.2.3 Measuring DNA methylation (Study III)

3.2.3.1 Illumina 450K array

Methylation was investigated for >485,000 CpG sites with the Infinium HumanMethylation450 BeadChip array according to manufacturer's guidelines (IIllumina). This was done by the core facility BEA, Bioinformatics and Expression Analysis, at Novum, Karolinska Institutet. DNA samples from 24 brain biopsies and six DNA samples from one *in vitro* experiment (3 mock and 3 HHV-6B infected Molt-3 cultures harvested 3 dpi) were diluted and sent for analysis. Files were received from BEA containing normalized, preprocessed, QC checked and analyzed values. Both beta and M values were obtained but the M values were used as they are more accurate to use for statistics compared to beta values [153]. As always when performing multiple comparisons in the statistical analysis, the statistics need to be adjusted for that. In study III, the Benjamini-Hochberg procedure was applied and a false discovery rate (FDR) <0.05 was regarded as significant.

Annotations were made against the genome version GRCh37/hg19, and this version was used for obtaining chromosome lengths and when investigating the data.

3.2.3.2 Bisulfite pyrosequencing

Results from large arrays, like the Illumina array used in this study, should always be technically validated in order to trust the obtained results from the array. Therefore, significant CpGs with large difference in M value were selected for validation using bisulfite pyrosequencing on new triplicate samples. Primers for five regions were designed in PyroMark Assay Design 2.0 (Qiagen), which would in total give methylation information about 9 CpGs from the Illumina array and 16 additional CpGs present in the analyzed regions. To investigate DNA methylation with the pyrosequencing technique, the template DNA first has

to be bisulfite converted. This was done using EZ DNA Methylation-Gold™ Kit (Zymo Research) according to manufacturer's protocol. This procedure will convert all unmethylated cytosine residues to uracil, but leaves 5mC unaffected. When sequencing the DNA on the PyroMark Q96 ID machine (Qiagen), this change in nucleotide will tell if the specific position is methylated or not.

The ordered primers were first optimized for the best annealing temperature. As template, 20ng bisulfite converted Molt-3 DNA was amplified according to manufacturer's protocol (Qiagen) but using three different annealing temperatures in the PCR program. The best temperature was chosen using agarose electrophoresis looking for the sharpest band on the gel. All primer pairs yielded sharp bands and were regarded as suitable to use.

In the next step a "bias test" was performed, i.e. % methylation was investigated with pyrosequencing on amplified DNA with known methylation status (Qiagen). In this step, several CpGs had to be excluded. For example, the methylation status on CpGs in the analysed DOC2B region did not yield correct results (Figure 9A). This non-linear, incorrect methylation status was also confirmed in a second experiment using more dilutions in the 50-100% methylated region (data not shown) and this primer pair was therefore excluded from the panel. This is a perfect example why the bias test was important, since usage of these primers would give unreliable results. Also, the read was shortened for one of the VPS53 regions as the read was long and only the first two CpGs "Passed" in the machine quality control and yielded expected % methylation (Figure 9B). Primer sequences of the included primers are stated in Paper III.

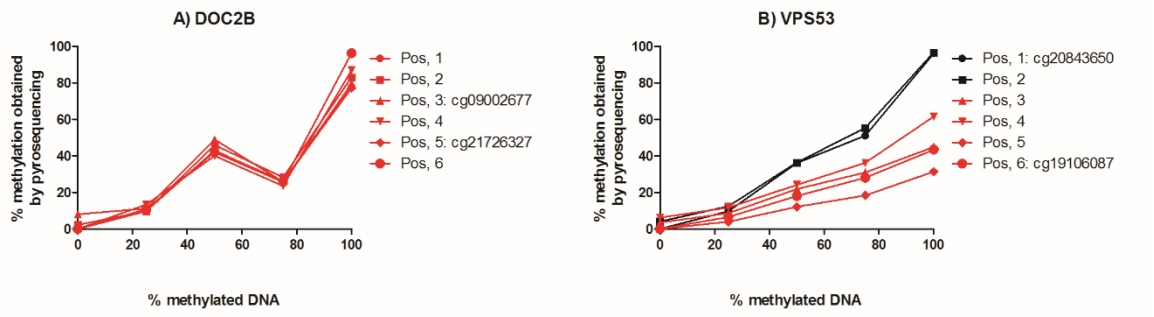


Figure 9: Bias test of bisulfite pyrosequencing primers. Primers specific for regions in the A) DOC2B B) VPS53 genes. Red color indicates that these CpG sites were excluded from further analysis.

When optimization was done, triplicate mock and HHV-6B infected Molt-3 samples were analyzed with the optimized primers. One CpG was followed in triplicate samples over time.

3.2.4 Measuring gene expression (Study II – III)

We wanted to investigate the expression of genes with HHV-6B induced change in DNA methylation. However, it is not sufficient to investigate only the gene of interest as that will highly depend on the input of cDNA. The most common way of presenting gene expression is by "relative gene expression" where the gene of interest is normalized to a reference gene, i.e. a stable gene not affected by the experimental condition. As we did not know what reference gene that could be regarded to be stable during HHV-6B infection, we had to figure that out.

All qPCR analyses were performed using TaqMan™ Gene Expression Assays according to manufacturer's protocol in 384-well plates on a QuantStudio™ 7 Real-Time PCR System (Applied Biosystems). Each well contained 20 ng cDNA (except in efficiency tests) in a total volume of 12µl. All plates included 'no reverse transcription' and 'no template' control.

3.2.4.1 Study II

In order to find a suitable reference gene to use in Study III, eight genes previously investigated during HHV-6A infection [154] were investigated for suitability to use as a reference gene during HHV-6B infection.

When investigating the expression of one gene in comparison to another, it is of highest importance that the PCR efficiency is similar for the two genes. If one gene amplifies the product at a higher rate compared to the other, the difference between them will vary with the cDNA concentration used. The eight investigated reference gene assays had very similar efficiency (Average = 86% ±2%; 2% CV), calculated based on the slopes visualized in Figure 10. The efficiency is generally accepted if 90-110%, but the efficiency is highly dependent on e.g. the pipet used and we believe it is more important that the assays are similar than to be exact within the suggested range.

$$Efficiency\ (E) = \ 10^{-1/slope} - 1$$

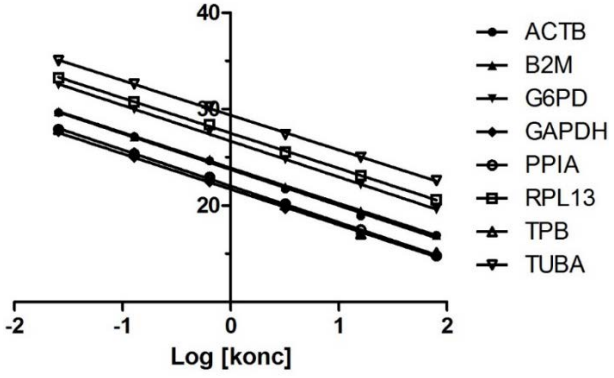


Figure 10: PCR efficiency of investigated reference genes. Log10-transformed cDNA concentration plotted against the raw C_T values. cDNA converted from total RNA extracted from uninfected Molt-3 cells was used as template. Efficiency was calculated using the stated formula.

As all eight TaqMan assays had similar efficiency, and did not amplify the negative controls, they were all included for investigation of stability during HHV-6B infection. In that step, 5 uninfected (0 dpi), 5 mock- and 5 HHV-6B-infected (3 dpi) samples were used. Stability of the genes was investigated using four different methods. These were the $2^{-\Delta\Delta CT}$ method [155] (Formula 2) and the three algorithms BestKeeper [156], GeNorm [157] and NormFinder [158].

$$\Delta C_T(calculated\ for\ each\ sample/calibrator) = C_T\ target\ gene - NF$$

$$\Delta\Delta C_T(calculated\ separately\ for\ each\ gene) = \Delta C_T\ sample - \ \Delta C_T\ Calibrator$$

$$Relative\ expression = \ 2^{-\Delta\Delta CT}$$

Formula 2: $2^{-\Delta\Delta CT}$ method used to calculate relative expression of all investigated reference genes in study II. C_T = threshold cycle, i.e. the value reflecting how many amplification cycles that are needed to reach the set threshold in the PCR machine. NF = normalization factor, the geometric mean of all C_T values obtained for all reference genes (except TBP as this gene was not suitable as reference gene). ΔC_T Calibrator = Average ΔC_T of the 5 uninfected 0 dpi Molt-3 samples.

As expression of TBP was found to be altered by HHV-6B infection 3 dpi, we wanted to monitor this expression over time. To do that, we analyzed expression of this gene in triplicate samples obtained from 5 time points between 0 to 6 dpi. In this additional experiment, PPIA was used as reference gene as it was determined to be the most stable in expression during HHV-6B infection.

3.2.4.2 Study III

Expression of the four genes *DOC2B*, *RPH3AL*, *RFLNB* and *VPS53* was investigated. As described above, efficiency was first evaluated. Efficiency of the RFLNB, VPS53 and RFLNB assay was very similar to the efficiency of the PPIA assays (≤5% CV), indicating that PPIA can be used as a reference gene when investigating RFLNB, VPS53 and RFLNB expression. However, the DOC2B gene was very low expressed and the obtained efficiency was not the same as for PPIA (21% CV, probably due to uncertainty of high C_T values). As uninfected Molt-3 cells did not obtain any C_T values with the TBP gene expression assay, the accuracy of this gene was not further investigated.

Based on the results presented in Study II, PPIA was used as a reference gene in Study III. Five mock and five HHV-6B infected (3 dpi) samples were analyzed. In addition, VPS53 expression was further investigated over time in a separate experiment using new triplicate samples obtained day 0, 1, 2, 3 and 6. Relative expression was calculated with the $2^{-\Delta\Delta CT}$ method using the average ΔC_T for mock (3 dpi) or untreated (0 dpi) as calibrator.

3.2.5 Investigation of virus integration (Study III)

To investigate if the induced hypomethylation at 17p13.3 could be associated with HHV-6B integration into the telomeric region of chromosome 17p, a PCR method was used to amplify one of the recombination sites on one side of the virus. With one primer binding to 17p and the other primer binding to HHV-6B DR_R, amplification of a ~1.5kb product indicates integration (Figure 11).

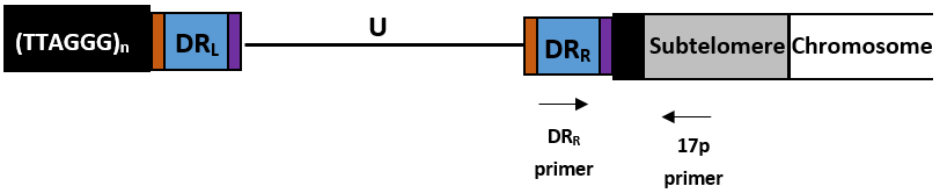


Figure 11: Primer location. Approximate location of primers used in integration PCR. Black = telomere region, grey = subtelomere. DR_L – U – DR_R represent the virus genome (see section 1.1.1.3 for more info). Information adapted from [66]. Figure not in correct scale.

Primer design was copied from Arbuckle et al. [66] but optimization was needed in order to ensure that the most suitable conditions were used. In the optimization step, 100ng DNA was used as template (mock or virus-infected Molt-3 cells 6 dpi). Three different annealing temperatures and two different primer concentrations were used, and amplified products were visualized with agarose gel electrophoresis. A ~1.5kb DNA fragment was observed in the virus-infected sample, but not in the uninfected sample, when using the primer concentration 0.5 µM and an annealing temperature of 60 °C, hence this was the chosen condition in the next experiment.

To investigate integration in our material, triplicate DNA samples of uninfected (0 dpi and 6 dpi) and HHV-6B infected (1, 2, 3 and 6 dpi) Molt-3 cells were analyzed.

3.3 STUDY IV-V

3.3.1 Study participants

All individuals included in Study IV and V were already included in other, ethically approved, studies. The aim of Study IV and V, i.e. to investigate risk factors for MS disease, was in line with the aim of the studies to which the study participants were recruited. All study participants provided written informed consent and the studies were performed according to the ethical standards of the Declaration of Helsinki.

3.3.1.1 Study IV

The study participants (446 MS patients and 487 controls) included in study IV were all part of the Epidemiological Investigation of Multiple Sclerosis (EIMS, [92]) study cohort. Treatment data was obtained for 368 MS patients from the national Swedish MS registry [159]. For detailed information regarding the included cohorts, see Table 1 in Study IV.

3.3.1.2 Study V

Two different patient cohorts were used in Study V, one with samples taken during MS disease and one where the samples analyzed had been collected before MS diagnosis.

The established MS cohort included the 916 EIMS participants analyzed in Study IV, but the number of study participants was greatly increased in Study V. In total, 8526 MS patients and 6932 controls matched for age, gender and residency were included from the EIMS study (n=5266), Genes and Environment in Multiple Sclerosis (GEMS [160], n=8940), Immunomodulation and Mutiple Sclerosis Epidemiology study (IMSE [161], n=1152) and Stockholm Prospective Assessment of Multiple Sclerosis (SPASM/STOPMS [162], n=100) study cohorts (Table 1 in Study V).

The pre-MS cohort was a prospective case-control study on biobank samples drawn before symptom onset (Table 2 in Study V). The Swedish MS registry [159] was crosslinked with three Swedish microbiological biobanks. These biobanks contained the remainders of sera after clinical microbiological analyses performed at the University Hospitals of Skåne and Göteborg, and the Public Health Agency of Sweden. The pre-MS patient samples were collected from individuals less than 40 years of age who later developed RRMS (n=480). Individuals who did not develop MS served as controls (n=480). Controls were matched for biobank, sex, date of blood sampling and date of birth.

3.3.2 Generating data

3.3.2.1 Measurement of anti-HHV6A/6B IgG (study IV)

As no method was available for measuring antibodies specific for either HHV-6A or HHV-6B when study IV was planned, we chose to investigate the anti-HHV-6A/6B IgG response with a commercial anti-HHV-6 IgG Antibody ELISA Kit according to the manufacturers' protocol (Advanced Biotechnologies). The antigen in this ELISA was whole HHV-6B virion lysate, but as most of the viral proteins are very similar between HHV-6A and HHV-6B this assay measures antibody responses against both virus species.

All plasma samples (n=933) were run in duplicate wells and approximately equal number of case and control samples were analyzed on all plates. The titer results are given as normalized optical density (OD) ratios, calculated by OD_{sample} divided by $2xOD_{neg\ control}$. As suggested in the assay protocol, OD ratios ≤0.75 were regarded as negative and OD ratios ≥1.00 as positive. Borderline samples (OD ratio 0.76 - 0.99, n=36) were excluded from the antibody prevalence analysis but included when titers were analyzed. The positive control's OD ratio on all plates had an inter-assay CV of 13% indicating a stable assay.

3.3.2.2 Measurement of anti-p41 IgG and total IgG (study IV)

To investigate the specificity of the anti-HHV-6 antibodies, samples from 67 RRMS patients and 67 controls (Table 1B in Study IV) were further examined for IgG specific for the HHV-6A/6B early antigen p41 and for total plasma IgG levels. These samples were selected based on equal anti-HHV-6A/6B IgG titers and were matched for gender, HLA-A*02 status and smoking habits. In addition to the 134 samples, plasma samples from 8 MS patients and 8 healthy controls which were negative for anti-HHV-6 IgG were also investigated for total IgG. Commercial ELISA kits were used for detection of IgG against p41 according to manufacturer's protocol (Bioworld Consulting Laboratories). Results were obtained as OD ratios and interpreted as above. Total IgG was measured using Human IgG ELISA kits (ALP) according to protocol (Mabtech). All plasma samples were run in duplicate wells.

3.3.2.3 Measurement of anti-IE1A, IE1B and 101K IgG (study V)

In order to study the anti-HHV-6A/6B IgG response more in detail, a new method was used to measure IgG antibodies specific for viral epitopes selected to be as divergent between HHV-6A and HHV-6B as possible. This method is a multiplex Luminex assay that uses beads coated with recombinant glutathione s-transferase (GST) fusions proteins allowing simultaneous measurement of numerous IgG specificities at the same time. The exact assay procedure has been described in detail elsewhere [163] and simplified in Study V. Antibody responses against four different HHV-6 protein sequences were analyzed. These were the HHV-6A and -6B specific parts of the immediate early protein 1, IE1A and IE1B respectively, and HHV-6A and -6B specific parts of the structural protein 101K (HHV-6B) and p100 (HHV-6A). Due to low reactivity against p100, this epitope was removed from further analysis. A Luminex 100 analyzer was used to measure the fluorescent signal and the results are expressed as median fluorescence intensity (MFI).

3.3.3 Analysis of data

3.3.3.1 Cutoff for seropositivity

For the ELISA kits used in study IV, the cutoffs for seropositivity were included in the kit manuals. However, for the Luminex assay, we had to decide what cutoff values to use in order to determine the serostatus. This was done by correlating the Luminex MFI results with the ELISA OD results obtained in Study IV (n=916), presuming that the ELISA measures IgG antibodies specific for all viral epitopes and that these values were "correct". For each antigen, the serostatus cutoff was determined to be where the specificity and sensitivity was best. In addition, a 5% exclusion was added so that two cutoffs were used when measuring seropositivity. The samples with MFI above the higher cutoff were regarded as seropositive and the samples with MFI value below the lower cutoff were seronegative while samples with values in between the cutoffs were excluded from serostatus analysis.

3.3.3.2 Transformation

As the obtained antibody distribution was skewed in both Study IV and V, the antibody levels were transformed before statistical analyses with linear regression models were performed. This was done using the Box-Cox power transformation in STATISTICA 11 in Study IV and a Log10-transformation in R in Study V. As an example, the distribution of untransformed and Log10-transformed anti-IE1A IgG levels are visualized in Figure 12.

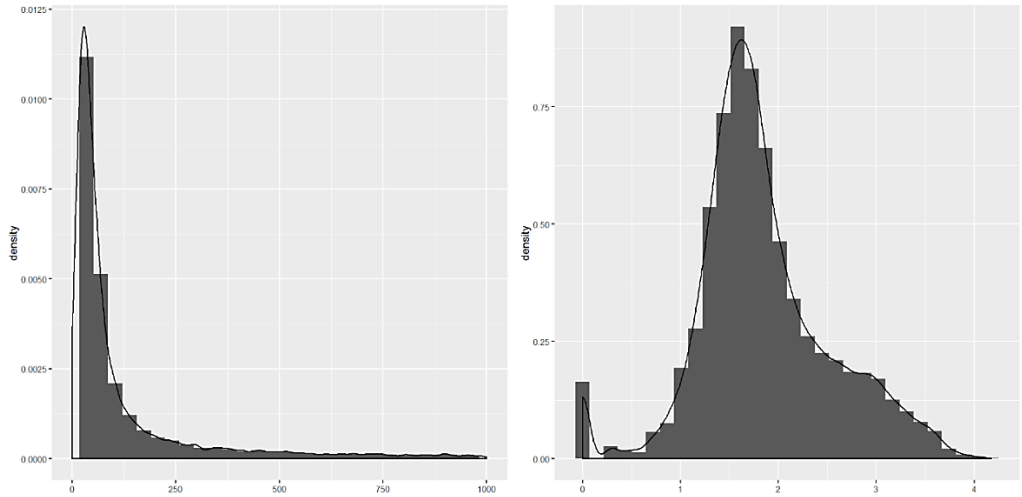


Figure 12: Histogram before and after Log10-transformation of anti-IE1A IgG levels. X-axis show MFI.

3.3.3.3 Statistics

The statistical analyses are similar in paper IV and V, but in paper IV nonparametric tests (Mann Whitney U or Kruskal-Wallis ANOVA and Pearson's chi-squared tests) were used as primary calculations and linear regression models as a way to confirm the results and to test the effect of potential confounders. In paper V, the regression models were the primary statistical measurement and nonparametric tests only performed as one sub-analysis. Also, in paper V the primary focus was on logistic regression models with serostatus as dependent variable, which was not used in paper IV. The main advantage with the regression analyses is that potential confounders can be added to the model and adjusted for. The cohorts were adjusted for slightly different variables as different information was available for use, but all models were adjusted for age at sampling and gender (see the articles for exact information). The threshold for significance was set at $p < 0.05$ in both paper IV and V.

The presentation of our logistic regression results focus both on the obtained p-values and the Odds ratios (OR). OR is often used to report an association between an exposure and an outcome. In study V the serostatus for each epitope was used as "exposure" and MS disease as an outcome (Formula 3). Together with the point estimate of OR, the 95% confidence interval (CI) is presented, which yields a good indication on the strength of the presented OR. If the 95% CI spans over the number one, the association is not statistically significant. In addition to the OR and 95% CI, p-values obtained from the logistic regression models were presented to show the significance of the association.

A) $$OR = \frac{\left(\frac{number\ of\ seropositive\ MS\ patients}{number\ of\ seropositive\ controls}\right)}{\left(\frac{number\ of\ seronegative\ MS\ patients}{number\ of\ seronegative\ controls}\right)}$$

B) $$y = b_0 + b_1X_1 + b_2X_2 + b_3X_3$$

$$OR = e^{b1}$$

Formula 3: Calculation of Odds ratio (OR). A) The formula for manually counting OR, without any covariates. B) Logistic regression and how to convert the beta value into OR. y=dependent variable, i.e. MS in this study, X= investigated independent variables, b_0 = intercept of the regression model, b_1 = slope of variable 1, e.g. serostatus. The interpretation of these examples would be: OR=1; Serostatus does not affect odds of MS disease, i.e. no association between seropositivity and MS disease. OR>1; Seropositivity is associated with higher odds of MS disease. OR<1; Seropositivity is associated with lower odds of MS disease.

3.3.3.4 GWAS

GWA studies were performed in order to investigate the genetic association with the three measured anti-HHV-6A/6B epitope specific IgG responses. Genotypes were previously determined using Infinium OmniExpress BeadChips (Illumina). In order to assure the quality of all genotyped SNPs, SNPs with <2% minor allele frequency (MAF), SNPs genotyped in <98% of individuals, and SNPs not in Hardy-Weinberg equilibrium among controls ($p<0.0001$) were removed from further analysis. In addition, individuals with >2% failed SNP genotyping, individuals with increased heterozygosity (>mean+2SD), related individuals, or individuals where the recorded sex differed from their genotype were removed from analysis. Also, a principal component analysis was conducted using Eigensoft [164] in order to control for population stratification, and in order to identify and remove all population outliers. After quality controls, data on approximately 600,000 SNPs for 6,808 MS cases and 5,671 controls from the established MS cohort was included in the GWASs.

Logistic and linear regression models for serostatus and antibody levels, respectively, were analyzed using PLINK v1.9 [165]. This was first done separately for MS cases and controls, followed by a meta-analysis.

3.3.3.5 HLA-imputation and association between HLA haplotypes and antibody responses

In order to determine association between the antibody responses measured in Study V and carriage of HLA alleles, SNP genotyping data was obtained from the MS Replication Chip. This chip contains approximately 90,000 SNPs chosen to densely cover the MHC region and because of previous association to MS.

HLA allele variants for MHC class I and II were imputed by the software HLA*IMP:02 [166] for 7,641 MS cases and 6,453 controls. Associations between HLA alleles and serostatus or antibody levels against each HHV-6 antigen were determined by logistic and linear regression models using R version 3.3.1. Associated alleles were combined into haplotypes using previously reported common haplotypes in the Caucasian population. Analyses were stratified by MS affection status and adjusted for gender and age at sampling.

4 RESULTS AND DISCUSSION

4.1 STUDY I

When doing experimental research with viruses, it is important to know if the virus batch one has is infectious or not, and how much of this virus batch to add to the cell culture experiments in order to have the infectivity of choice. As no standard methods for measuring HHV-6A/6B infectivity was available, we aimed at developing a correct, robust and easily interpreted assay.

We developed an HHV-6A specific $TCID_{50}$-method and compared different readouts of the plate to each other. As seen in Table 1, the different readouts used yielded similar $TCID_{50}$ values. However, high inter-assay CV was obtained using all readouts, indicating large variation in the $TCID_{50}$ assay itself. When comparing the $TCID_{50}$ method to the Inf U assay we observed similar variation within the methods (inter-assay CV 73% for $TCID_{50}$ with qPCR readout and 77% for the Inf U assay), indicating that a high variation might be expected when working with HHV-6A infection of HSB-2 cells.

The qPCR readout correlates well with IFA readout indicating a correct result when analyzing DNA as a readout for positivity. The intra-assay variation was lowest for the qPCR readout (9% CV). This means that when extracting DNA from the same plate two times with subsequent different PCR amplifications, the results became similar, indicating a robust readout. The ocular inspection had the highest intra-assay CV (45% CV), confirming our impression of a subjective readout.

Virus batch	$TCID_{50}$/ml Q-PCR	$TCID_{50}$/ml Ocular inspection	$TCID_{50}$/ml IFA	Inf U/ml IFA	HHV-6A DNA cop/ml
P17	1215 ±566 (x3)	771 ±466 (x3)	972 ±719 (x2)	nd	7.6e8 ±1.4e8 (x2)
P19	806 ±679 (x5)	465 ±408 (x5)	649 (x1)	2.5e4 ± 1.6e4 (x3)	16.0e8 ±1.3e8 (x2)
P21	6 ±8 (x2)	14 ±6 (x2)	nd	nd	7.7e8 ±0.15e8 (x2)
P27	7 ±6 (x3)	30 ±15 (x3)	13 ±3 (x3)	3.9e4 (x1)	nd
P17 UV-inact.	0 (x1)	0 (x1)	nd	nd	nd
P17 heat-inact.	20 (x1)	4 (x1)	nd	nd	nd

Table 1: Results from different titer assessments. Inf U = infectious units, nd = Not done

The P21 and P27 virus batches show very low $TCID_{50}$ values, indicating few infectious virions per ml. However, the DNA load of P21 was similar to the more infectious batches, indicating

that measuring DNA load does not have to correlate with virus titer. Also, the Inf U assay yielded similar values for P19 and P21 indicating that this IFA-based assay might have low sensitivity and not be suitable to use for low-titer batches.

4.1.1 Reflections

In study II and III, the $TCID_{50}$ assay with qPCR readout for HHV-6B was used (although the method is only validated for HHV-6A in Study I). To calculate MOI, i.e. the number of virus particles per cell, often used as a measurement of viral input in experimental settings, the $TCID_{50}$/ml first needs to be converted to number of viable virions/ml. This was in study II and III done by multiplying the $TCID_{50}$/ml with 0.69 (based on the Poisson distribution). The MOI 0.01 was used meaning 0.01 viable virions per cell. This may be an underestimation as >95% of the cells were infected 3 dpi. This suspicion can be augmented when looking closer at the relationship between the Inf U results and the $TCID_{50}$ results. The readout of the Inf U assay is infectious units (i.e. virions) per ml and should therefore, according to my way of converting be Inf U /ml = $TCID_{50}$/ml x 0.69. If the Inf U results for P19 are "correct", and the conversion can be made as stated above, the $TCID_{50}$ should have been approximately 36,000 $TCID_{50}$/ml. I am confident in the titer calculations made to obtain the $TCID_{50}$ value/ml and suspect that the possible underestimated titer value is most probably due to the conversion to MOI. When looking back at these calculations I think it is more correct not to convert $TCID_{50}$/ml to MOI, but instead specify the $TCID_{50}$ added to each experimental condition, although this titer unit is hard to interpret.

The Inf U assay is laborious with all IFA stainings and subsequent cell counting, but I believe an improvement of this assay could be a good option. One possible readout could be flow cytometry (FACS). However, this readout would need optimization to set criteria for positivity. Although our result of the Inf U assay indicate that it could not distinguish between our two tested virus batches, that might not be a problem if using more infectious virus batches or another readout. One major advantage of this assay is that the units obtained are easily interpreted.

However, I believe that the exact titer might not be so important as long as there is a measurement of infection within the study experiment (for example % infected cells). The variations between laboratories will be large anyway due to different viral strains, cells and other conditions. On the other hand, a reliable titer determining assay is needed as it is of highest importance to keep track of titers within a lab in order to repeat experiments etc.

In conclusion, I think that the $TCID_{50}$ method can be a good choice and that the qPCR readout is a correct and robust readout, especially when the assessor is not used to visual inspection of infected cells. However, the $TCID_{50}$ method might underestimate the titers and does not yield so easily interpreted titer units.

4.2 STUDY II

In order to measure relative gene expression in experimental studies like Study III, we first had to determine what reference gene to use for normalization in order to obtain reliable results. Ideally, several stable reference genes should be used, however, having more genes results in greater costs and less space on an analysis plate. In addition, the GeNorm analysis used demonstrated little increase in reliability when using one or two reference genes during HHV-6B infection. Therefore, we decided to use only one in future experiments.

We used four different analysis tools to determine reference gene stability and all gave similar ranking of the 8 investigated genes, with the exception for the standard deviation (SD) analysis in BestKeeper. This analysis relies on the assumption that the input cDNA is similar in all samples and that the expression of investigated genes should be stable, hence yield a low SD. This may be a good analysis when investigating for example a drug effect, but when working with infections this analysis should be interpreted with caution. As seen in Figure 13, we observed that the RNA load is increased in virus infected cell cultures, probably due to virus RNA being expressed. As the same amount of total RNA was transcribed to cDNA and used for expression analyses, the relative amount of the investigated reference genes is decreased in infected samples. Therefore a low SD of a reference gene indicates upregulation of this gene.

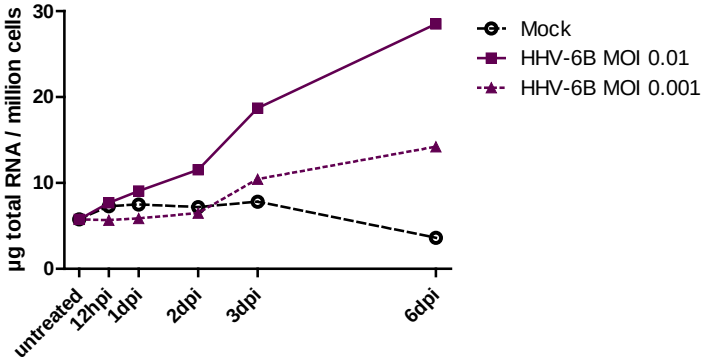


Figure 13: Average µg extracted RNA from 10^6 living cells over time in one experiment. Mean of triplicate samples. Total RNA measured with nanodrop and living cells were counted in hemocytometers under a phase contrast microscope.

The gene ranking was similar between the different analyses, and results from the $2^{-\Delta\Delta CT}$ method are presented in Figure 14. According to this analysis; peptidylprolyl isomerase A (PPIA), ribosomal protein L13 (RPL13) and β-actin (ACTB) are most suitable as reference genes when normalizing expression data in HHV-6B infected Molt-3 cells. This figure also clearly visualizes the expressional difference of TATA-box binding protein (TBP) observed with all analysis tools. If using TBP as a reference gene, it is highly plausible that other genes will falsely appear downregulated.

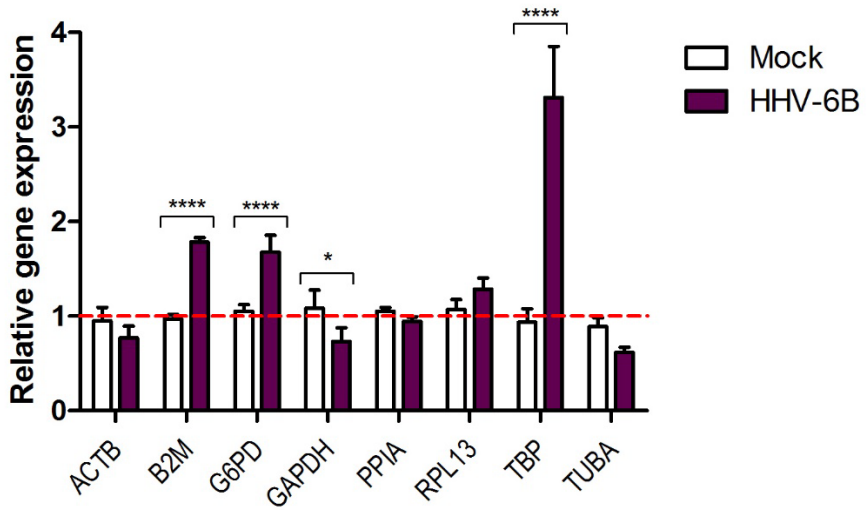


Figure 14: Relative expression ($2^{-\Delta\Delta CT}$) of the eight investigated reference genes. Expression in mock (n=5) and HHV-6B (n=5) infected cells (3 dpi) compared to uninfected cells (0 dpi). TBP is not included in the NF used for calculations. Statistics from a two way ANOVA is presented: * = $p<0.05$, **** = $p<0.0001$.

As TBP does not appear to be upregulated by other herpesviruses [154], it is likely a HHV-6B specific upregulation. The gene *TBP* is located close to the telomere at chromosome 6q (although not differently methylated by HHV-6B, data not shown) and the encoded protein binds DNA and can initiate transcription. If the observed expressional increase has any biological impact cannot be answered by our study. However, TBP has been found to co-localize in the cell nucleus with the HHV-6B viral U94 [167] and DR6 proteins [168]. U94 is a latency associated protein described in section 1.1.3 and DR6 is a protein that delays cells in the G_2/M-phase hence inhibit cell proliferation of host cells [169]. Also, TBP binding to DNA is increased during HHV-6B infection [168] and it is therefore highly plausible that the expressional increase observed in Study II is important in the HHV-6B biology.

In conclusion, PPIA was chosen as a suitable reference gene to use in Study III and it would be interesting to further study the interaction between TBP and HHV-6B.

4.3 STUDY III

Viruses have been suggested to affect DNA methylation in host cells in various ways (for more info see section 1.4.2), however no information was available on how HHV-6B or HHV-6A affects DNA methylation. We chose to work with HHV-6B as this virus has been associated to epilepsy (see section 1.3.2) and we wanted to investigate possible pathogenic effects of this virus caused by epigenetic modifications.

Using an Illumina 450K whole genome methylation array, we investigated approximately 450,000 CpG sites at the same time. This array revealed that HHV-6B induces hypo-

methylation, i.e. less methylation compared to controls, in the host cell genome (Figure 15). The distribution of hypomethylated CpGs did not appear random as it was primarily taking place close to the telomeres. This was most evident at the end of chromosome 17p where 34% (138/406) of the significant CpG sites were located.

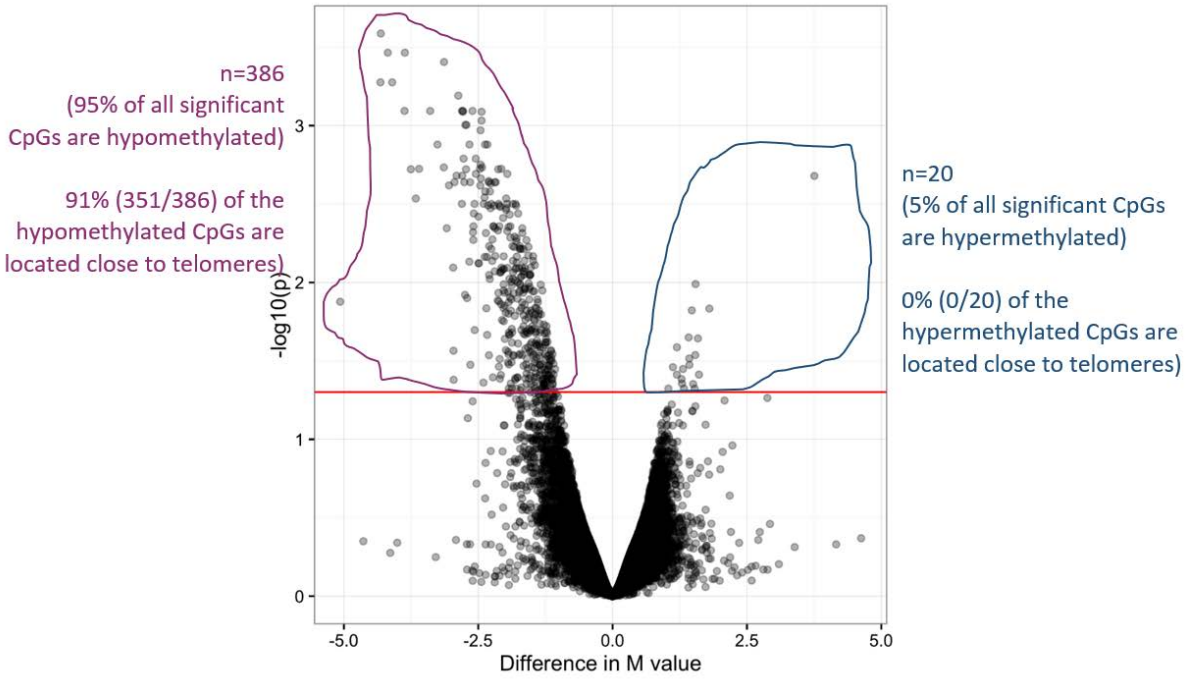


Figure 15: Volcano plot visualizing the Illumina 450K results. Each dot represents one CpG site. A negative M value interprets as less methylation in virus infected Molt-3 cells compared to uninfected cells 3 dpi (i.e. hypomethylation), a positive M value interprets as more methylation in infected cells (i.e. hypermethylated). Values above the red line are significant (FDR<0.05).

The observed HHV-6B induced hypomethylation was confirmed in new triplicate samples using bisulfite pyrosequencing. In addition, we wanted to investigate the temporal regulation of this epigenetic change. Methylation status of one CpG site in the *VPS53* gene (cg20843650) was followed over time and was found to be hypomethylated 2 dpi (Figure 16A).

In order to investigate if the observed virus-induced hypomethylation had any impact on gene expression, we chose to investigate expression of the four genes located in the hypomethylated region at 17p13.3: *DOC2B*, *RPH3AL*, *RFLNB* and *VPS53*. All of these four genes had higher expression level in HHV-6B infected Molt-3 cells compared to in mock-treated cells, indicating a functional role of the DNA methylation. The expression of VPS53 was followed over time and was found to be significantly upregulated already at 2 dpi (Figure 16B). This similar time line for DNA methylation and gene expression indicates an association between the two measurements.

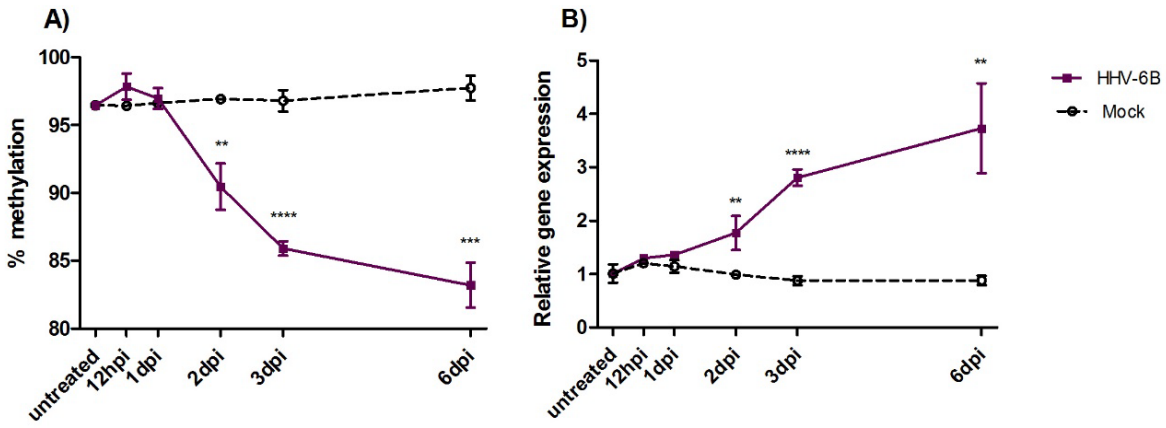


Figure 16: The effect of HHV-6B infection on the VPS53 gene over time. A) Methylation of cg20843650 (VPS53) measured with bisulfite pyrosequencing. B) Expression of VPS53. Mean and standard deviation of triplicate samples. ** = p<0.01, *** = p<0.001, **** = p<0.0001

As 17p13.3 has been reported to be an integration site for HHV-6B (see section 1.1.3), we suspected this hypomethylation to be involved in HHV-6B integration. To test this hypothesis we used a previously developed PCR method that amplifies one of the integration sites. We could observe amplification of a product with anticipated size in all triplicate samples analyzed at 3 dpi and 6 dpi, confirming the finding by Arbuckle et al. [65] who observed HHV-6A integration already during lytic infection. This time frame indicates that the observed locus-specific hypomethylation might play a role in the integration process.

We could not observe a difference in DNA methylation in the *in vivo* material. This could be due to a transient hypomethylation. It is also likely that we did not have power to detect a difference due to few samples, heterogeneity within one sample (one brain biopsy contains many different cell types with different epigenetic landscapes) and large heterogeneity between individuals. Also, the percent infected cells was very low (data not shown). Taken together, even though the virus might induce a difference, it will probably be masked by all uninfected cells in the tissue or by the background noise making it hard to pick up a virus-induced signal in a small material. If we would have used the same numbers as for the GWAS study in study V (n= 6808 + 5671), the results could have been different.

This is the first study to investigate the effect of HHV-6B on DNA methylation, and these results open up a new dimension of the HHV-6B integration process. If I were to continue this project, I would first try to do a long term experiment to see if this hypomethylation is transient or not, answering the question if the hypomethylation is specific for the integration process only. Long term culture of infected cells can however be problematic as most of the cells will die during the lytic phase of infection. Secondly, I would investigate the TET activity, i.e. the de-methylating enzymes (Figure 7), during HHV-6B infection. However, a global change in TET levels would not explain the region-specificity observed in this study.

It would be very interesting to know the mechanism behind the observed site-specific hypomethylation. Is there a virus protein that interacts with proteins located at telomeres or subtelomeres? Even though the TMRs in the virus genome may explain the specificity for 17p [76], it does not explain the 17p-specific hypomethylation. Although there is much more to de done until we fully understand this process, this is a first step on the way.

4.4 STUDY IV AND V

Study IV and V investigate the anti-HHV-6A and/or HHV-6B IgG response in MS patients and controls and investigate the association between the measured antibody responses to other genetic and environmental factors.

The main difference between the two studies is the antibody epitopes. The antibody measurement in Study IV measured all antibodies that reacted against HHV-6B lysate, while in study V we selected and analyzed only antibodies specific for selected protein epitopes. As seen in Figure 17 there is not a strong correlation between the ELISA results and the Luminex results. This is most likely due to that they measure different antibody specificities; The ELISA potentially detects all IgG antibodies specific for any virus proteins present in the HHV-6B virion while the Luminex assay measures antibodies specific for selected epitopes. The low correlation indicates that an epitope-specific response is masked in the pool of all antibodies against the virus.

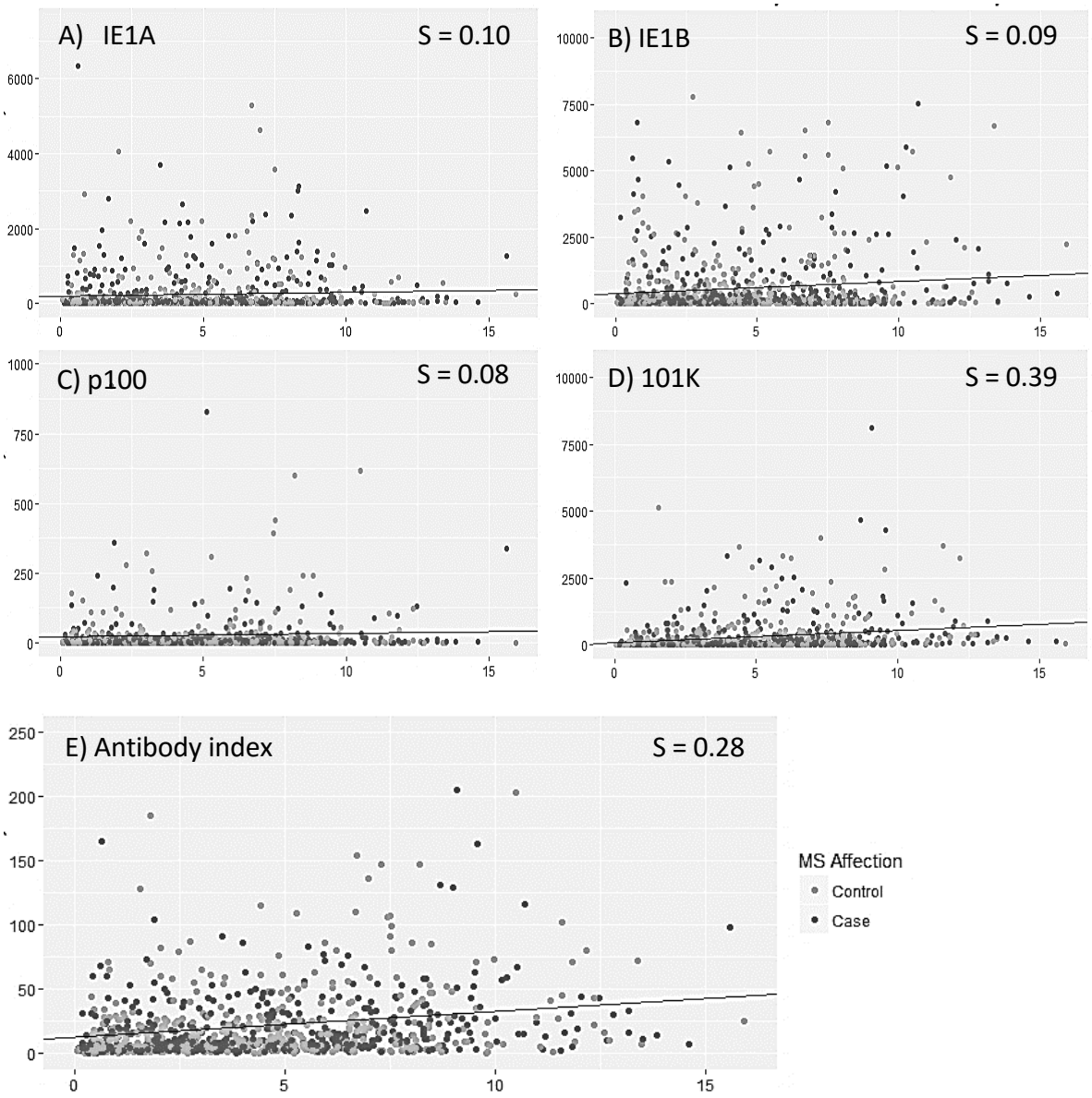


Figure 17: Correlation between antibody measurements in Study IV and V. X-axes display the obtained OD ratios in Study IV and the y-axes show MFI values for A) IE1A, B) IE1B, C) p100 and D) 101K obtained in Study V. X-axis for E) display "Antibody index"; an index based on sum of all 4 antigen divided by median value. S = Spearman correlation coefficient. Relationship also visualized with regression line. Note that the y-axes have different range. Data was available for 916 individuals.

4.4.1 Association to MS

When investigating anti-HHV-6A/6B IgG against HHV-6B lysate no difference was observed between MS patients and controls. However, when dissecting the antibody response we found, for the first time, an increased anti-IE1A and 101K IgG response in established MS patients compared to controls. In the opposite direction, lower IgG response against IE1B was associated to MS disease in women.

In order to investigate if the difference in antibody response precedes MS onset, the HHV-6 epitope specific antibody response was investigated in individuals who later developed MS. Interestingly, IE1A serostatus and levels were associated with MS risk, as were 101K IgG levels.

This indicates that HHV-6A, and possibly also HHV-6B, is associated with risk of developing MS. In fact, the highest OR was observed for IE1A seropositivity in the youngest age group in the pre-MS cohort (Figure 18), indicating that the acquisition of HHV-6A might have a triggering effect on MS disease development.

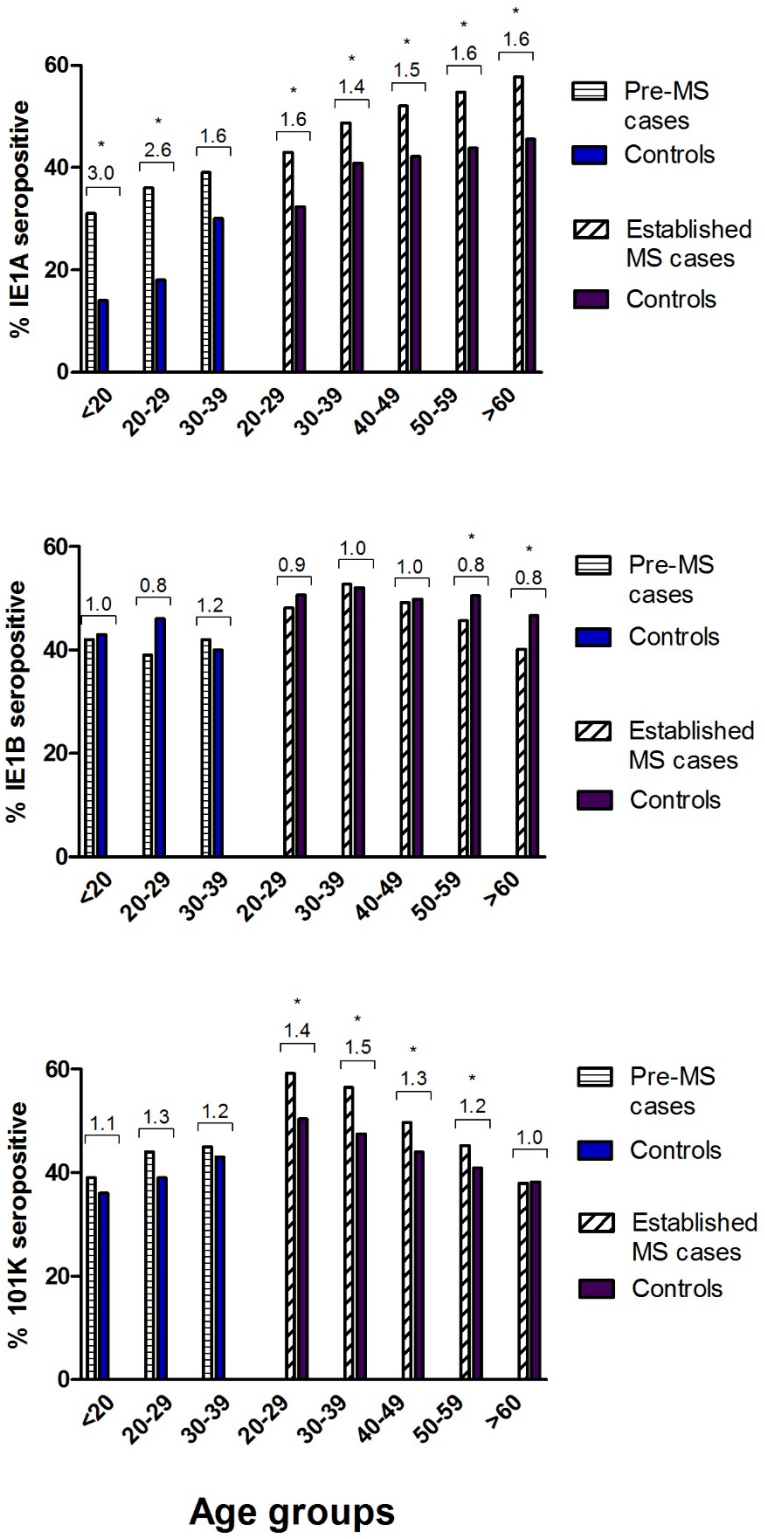


Figure 18: Seropositivity in different age groups. ORs are given for all age groups and * indicates a p-value less than 0.05.

As relapsing remitting MS and progressive MS have different diseases characteristics, with the RRMS course being more inflammatory (see section 1.3.1), the difference in antibody levels between these disease courses was investigated. Unfortunately, only the disease course in 2014 was currently available. We compared RRMS patients to primary progressive MS (PPMS) patients. Interestingly, as seen in Figure 19, the anti-IE1A IgG levels is higher while the anti-IE1B and 101K IgG levels are lower in PPMS compared to RRMS patients. Important to notice is that this can be due to the higher age in PPMS patients.

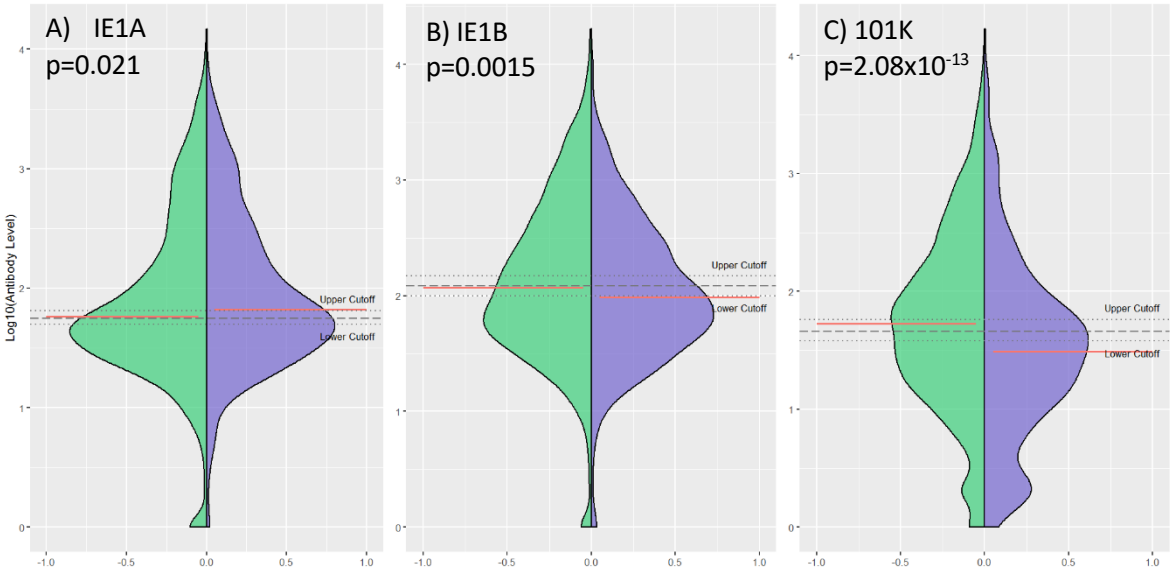


Figure 19: Antibody levels in RRMS and PPMS patients. Log10-transformed MFI values for IgG levels against A) IE1A, B) IE1B and C) 101K. Median value indicated with red line. Green = RRMS (n=5052, median years of age=41), Purple = PPMS (n=534, median years of age=60). Cutoff values indicated with dotted lines. p-values calculated with Wilcoxon rank-sum test.

4.4.2 How to interpret antibody data?

4.4.2.1 Cutoff for serostatus and seropositivity results

When looking at the Log10-transformed distribution of antibody levels (Figure 19), it can give the impression that the cutoff is set a bit arbitrary, but it is important to remember that these graphs do not show the original distribution. As seen in Figure 12 and 17, most of the untransformed MFI values are low. Another way to determine a statistical cutoff can be to use the median MFI among controls [96], which would yield a similar cutoff as set today (Figure 1 in Study V). However, the only way to determine true seropositivity is to use control sera with known serostatus. As we do not have a validated cutoff for determining positivity the exact % seropositive individuals should be interpreted with caution. However, as seen in Table 2, the combined seropositivities for all three epitopes measured in study V are in the range of 75-100% that has previously been reported as HHV-6A/6B IgG seropositivity in the general population [9, 11, 60-63].

Epitope	Virus species	MS Cases	Controls	TOTAL
HHV-6A/6B #	HHV-6A/6B	89.1	90.4	89.8
IE1A/IE1B/101K *	HHV-6A/6B	83.3	79.0	81.4
IE1A *	HHV-6A	53.1	43.7	48.9
IE1B/101K *	HHV-6B	70.8	69.8	70.3
IE1B *	HHV-6B	47.4	50.1	48.7
101K *	HHV-6B	49.8	44.3	47.3

Table 2: Percentage seropositive in Study IV and V. # = Study IV: ELISA according to manufacturer's protocol. * = Study V: Luminex assay using single cutoff on the Established MS cohort. When several epitopes are stated (e.g. IE1B/101K) this indicates positivity for at least one of the epitopes.

As seen in Figure 20, the IgG responses against the two HHV-6B epitopes IE1B and 101K do not correlate, indicating that individuals generally do not seem to have antibodies specific for both IE1 and 101K at the same time. This together with the data in Table 2 and the low correlation between anti-HHV-6A/6B IgG and the epitope-specific antibodies visualized in Figure 17 indicate that one protein sequence might not be sufficient for determining infection status in one individual.

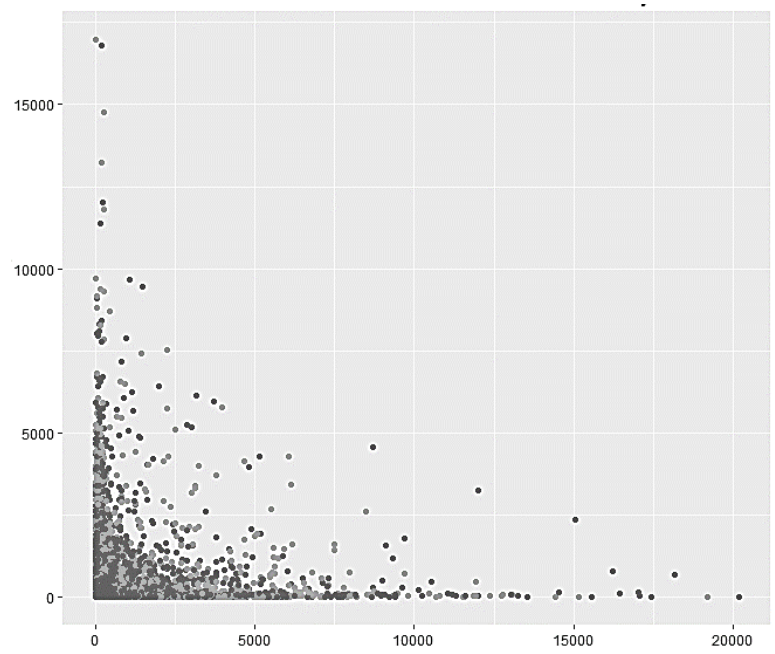


Figure 20: Correlation between IE1B (x) and 101K (y). One dot represents MFI values from one individual. Light grey = Control, dark grey = MS cases. Both the IE1B and 101K epitopes are selected from the HHV-6B genome.

4.4.2.2 What does an antibody response mean?

A strong antibody response in one individual can be interpreted in different ways. It can be interpreted as high viral burden in this individual hence an association of serological response to disease would indicate that infection or recurrent reactivations with this virus is associated to the disease. It can also be interpreted as a good immune response with the ability to keep the latent infection in place, hence whenever reactivations will occur the infection is well controlled by the immune system in this individual. In this case, association between an autoimmune disease and an increased serological response would indicate that the immune system may induce other detrimental effects when the anti-viral response is elicited. It is also likely that an antibody response can reflect both scenarios, where a high antibody level can reflect high viral load and a good control of the virus.

During infection, the viral IE1 proteins are located in the host cell nucleus. As seen in Figure 5, antibody responses are supposed to be elicited against extracellular epitopes (e.g. virus glycoproteins). If an antibody response is elicited against nuclear proteins, can this be a clue to virus pathogenesis or disease pathology? Other nuclear virus proteins give rise to similar responses. For example, a stronger immune response has been observed against the HHV-6A/6B protein p41 in MS patients [170]. The p41 viral protein is located within the nucleus and is involved in transcription [168, 171], suggesting a similar exposure to the immune response as IE1A and IE1B. Also, EBNA-1 elicit a stronger immune response in MS patients compared to in healthy individuals [172]. Necroptosis is a form of immunogenic programmed cell death where cell swelling results in rupture of cell membrane and release of intracellular components into the surrounding tissue (reviewed in [173, 174]). Can the anti-nuclear virus antibodies reflect that MS patients are more prone to get virus induced cell necroptosis and therefore more nuclear antigens in the extracellular space? This cannot be answered in this setting but the hypothesis has some support in literature. First, necroptosis has been demonstrated to be involved in MS; markers of necroptosis were found in MS lesions and TNF-induced necroptosis mediates oligodendrocyte degeneration [175]. Secondly, it has recently been discovered that the intrathecally produced antibodies characteristic of MS patients often are directed against ubiquitous intracellular proteins [176], indicating tissue destruction in the brain. However, the increased IE1A and 101K responses in MS might be a consequence and not a cause of tissue destruction. In favor for a causative role, supernatant from HHV-6A, but not HHV-6B, infected cell cultures has been observed to induce caspase-independent cell death (e.g. necroptosis) in oligodendrocytes *in vitro* [177].

4.4.2.3 anti-p41 IgG results

The high p41 seropositivity (100% among HHV-6A/6B seropositive individuals) observed in Study IV is surprising and not in line with previously reported 28% (20/72) seropositivity among controls and 69% (11/16) seropositivity among MS patients [170]. Unfortunately, we

did not analyze any negative anti-HHV-6A/6B sera which would have given an indication of the assay specificity. When we bought the ELISA kit, it was newly launched on the market, and now it is no longer available. The result from this assay should be interpreted with caution.

4.4.3 Associations to gender and age

Females were observed to have significantly higher anti-HHV-6A/6B IgG levels compared to males (Study IV). This gender difference was revealed to be epitope specific and it was primarily anti-101K IgG levels that were increased in females and not anti-IE1A/B levels (Study V) nor total IgG (Study IV). If this is dependent on different virus biology in the genders or a difference in how females and males respond to different virus epitopes cannot be answered in these studies. However, female sex has been associated with increased acquisition of HHV-6B in children [8], and it is possible that the anti-HHV-6A/6B IgG and anti-101K IgG responses reflect this difference in primary HHV-6B infection.

Interestingly, the seropositivity against the HHV-6A epitope IE1A increases with age while this pattern was not observed for the HHV-6B epitopes IE1B and 101K (Figure 18). In fact, the seropositivity against 101K tend to decrease with age in the Established MS cohort. This indicates that HHV-6A acquisition occurs during the time period investigated while primary infection of HHV-6B happened earlier, probably during childhood. This difference between the two viruses has been suggested previously in literature [16].

4.4.4 Genetic associations to anti-HHV-6A/6B epitope specific IgG responses

4.4.4.1 Association to Class II HLA genes

The absolute strongest genetic association to HHV-6A/6B IgG responses was observed between anti-IE1A antibody response (both serostatus and serolevels) and SNPs in the MHC region (Figure 21). The strongest association was found between HLA-DRB1*13:01 carriage and IE1A serostatus (OR 1.52, $p=3x10^{-15}$). Carriage of DRB1*13:01 has previously been associated to stronger response against Hepatitis B vaccination [178] and this allele has been suggested to code for amino acids in the HLA-DRβ1 specifically important for anti-EBNA-1 IgG response [146]. This suggests a role for MHC class II molecules encoded by this allele in presentation of many viral peptides and a role in eliciting anti-viral IgG responses. Even though a similar manhattan plot peak at chromosome 6 has been observed for EBNA-1 response [145, 146], the most associated SNPs previously reported for anti- EBNA-1 response was not associated to anti-IE1A response in our study.

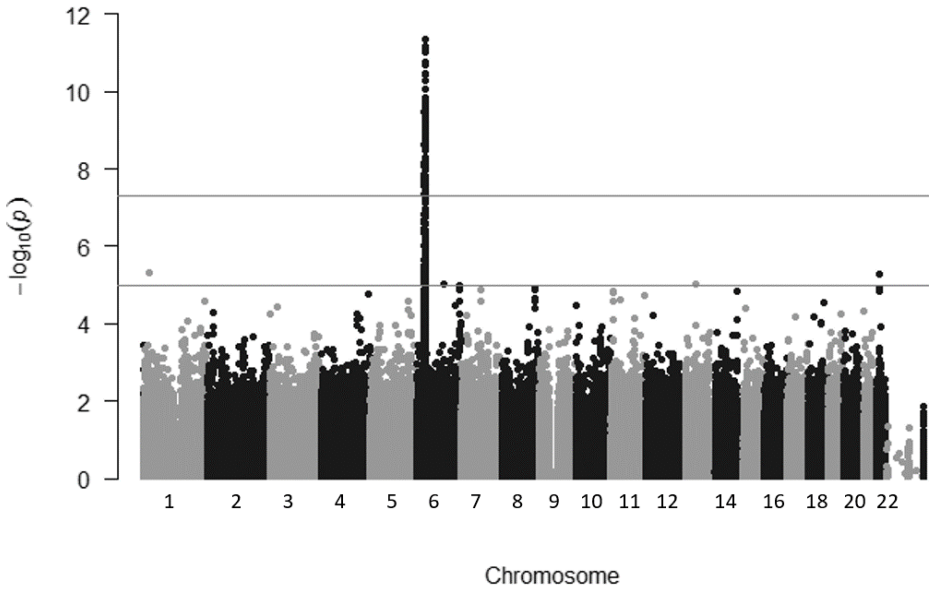


Figure 21: Manhattan plot visualizing GWAS results for Log10-transformed IE1A IgG levels. Both MS and controls included in the analysis. Each dot represents the p-value of one SNP. The peak at chromosome 6 shows significant SNPs located in the MHC locus. Line set at $p=5x10^{-8}$ indicating GWAS significance, and line set at 10^{-5} indicates suggestive association.

Antibody response against IE1B and 101K did not show such strong HLA peaks as the anti-IE1A response. One of the strongest associations for IE1B serostatus was with the HLA allele HLA-DRB1*07:01, however only significant in controls (OR=1.44, $p=5x10^{-7}$). This allele showed the strongest (negative) association to EBNA-1 IgG levels in a previous study [146].

Carriage of the MS risk allele HLA-DRB1*15:01 was not associated with a difference in anti-HHV-6A/6B IgG levels but with higher anti-IE1A IgG levels in MS patients (β=34, $p=1.1x10^{-12}$) and lower anti-IE1A IgG levels in controls (β=-35, p=0.01). If the association between this allele and increased anti-IE1A IgG response exclusively in MS cases reflects a connection between HHV-6A and MS cannot be answered in this setting. However, it indicates that carriage of this allele does not increase the anti-IE1A response *per se*, but that something else present in MS patients but not in controls, is needed for eliciting this response.

The finding that the three different viral epitopes investigated in Study V are genetically associated with different HLA alleles is interesting and probably reflects differences in both protein sequences, function of the proteins and different species origin. The IE1A and IE1B protein sequences are from the same protein but coded by the different virus species, and the IE1B and 101K epitopes are from different proteins coded by the same virus species. Even though not all individuals respond to all epitopes, the polymorphism within HLA genes seems to ensure that most individuals respond to at least one epitope.

4.4.4.2 Association to Class I HLA genes

Carriage of the MS protective HLA*A-02 allele was associated with lower levels of anti-HHV-6A/6B IgG (Study IV). In study V, HLA*A2:01 carriage was associated with a lower anti-101K but higher anti-IE1A IgG response.

B cells are activated through MHC class II (see section 1.2), so why are antibody responses associated to MHC class I molecules? It could be an indication of a better virus control by CD8+ cells. Naïve CD8+ T cells need to recognize MHC class I associated peptides together with costimulatory signals from an APC in order to become effector CTLs. Also, cells constantly present peptides from inside the cells on MHC class I molecules. It is therefore beneficial for an immune response against the virus if the MHC molecules of one individual are prone to bind and present virus-derived peptides. In line with this idea, 101K peptides are presented on HLA-A*02:01 encoded MHC class I molecules on HHV-6B infected cells and these infected cells are recognized and killed by CD8+ T cells [179]. If CTLs clear infected cells, the viral burden of the individual will be decreased, possibly explaining the lower levels of anti-HHV-6A/6B and anti-101K IgG levels in individuals with the HLA*A2:01 allele.

In further support of an importance of MHC class I associated regulation of serological response to 101K, we found a genetic association between anti-101K IgG response and 3 SNPs in *LILRB2,* a gene coding for the leukocyte immunoglobulin like receptor B2 (LILRB2). This protein is expressed on APCs and is associated to the APC's MHC class I molecule where it sterically competes with the CD8 binding [180, 181]. LILRB2 binds to different HLA-encoded MHC class I molecules with different binding strength and a strong binding leads to less T cell responses against HIV-1 [182]. Interestingly, the HLA-B*40:01 allele was associated to lower anti-101K IgG response (OR=0.79, β= -53), and this allele has strong binding strength to LILRB2 [182]. The functional role of the three associated SNPs in *LILR2B* is not known, but the dual association of LILRB2 and HLA-B*40:01 with anti-101K serological response may indicate a role of CTL mediated clearance of virus as an important regulating factor.

4.4.4.3 HLA association with anti-HHV-6A/6B IgG (Study IV)

A GWAS was also done on the material in Study IV, although the results were presented separately (not published manuscript) and not included in Paper IV. Due to the low number of included subjects, this study could have been underpowered to detect genetic associations to anti-HHV-6A/6B IgG levels and serostatus. However, there was no peak at Chromosome 6. The same was true for the GWAS study by Rubicz et al. [145] where they used the same antibody ELISA as we did in Study IV and observed no association to SNPs at chromosome 6. This highlights the difference in HLA-associations observed for the different epitopes, suggesting that when measuring a mix of all epitope-specific antibodies, there will be no GWAS signal.

4.4.5 Future perspectives in relation to Study V

The most important analysis to be done is to run control serum from individuals with known infection status so that we can conclude if the antibodies binding to the HHV-6A and HHV-6B specific sequences in the Luminex assay truly pick up HHV-6A and HHV-6B specific immune responses. Until now, we have not been able to find such samples but a collaboration has been initiated with Professor Yoshikawa in Japan and samples obtained from children with primary HHV-6B infection will be sent for analysis. If these samples do not show reactivity against IE1A it will at least indicate species specific detection. Also serum from macaque monkeys infected with HHV-6A or HHV-6B will be obtained through a collaboration with Dr. Jacobson in the USA. Important to remember is that a negative result can be because the patient/animal did not mount a response against this specific peptide. This is not unlikely as we show that individual tend to respond differently against different peptides. At this point in time, we cannot know if the peptides used actually measure species-specific responses.

The reactivity against p100 was very low in our material and these results were interpreted as this peptide not working properly in the assay. As we did not have any controls to suggest if these results were low by nature or by an assay artefact, these results were not included in further analysis. However, as Higashimoto et al [55] also found a low reactivity against p100 but strong reactivity against 101K, our p100 results might be true. Depending on the control results, the already measured anti-p100 IgG response may be examined more in depth until final conclusion can be made.

At the moment, the presented disease courses in Study V were the disease courses they had in the year 2014 and not at sampling. Although many patients had the same disease course at sampling and in 2014, this information needs to be updated (which will take a while). And when correct disease courses are known, the difference between RRMS and the progressive forms of MS will be further investigated.

Study IV demonstrates a difference in anti-HHV-6A/6B IgG levels between smokers and non-smokers. Smoking data for all patients included in Study V was not available but is to be obtained. When this data is obtained, it would be interesting to see if smoking affects only some epitope-specific IgG responses or if it the decreased anti-HHV-6A/6B levels induced by smoking can be reflected in a general decreased antibody production.

Since we see that HLA influences response against the different epitopes we need to rule out that the association we see between for example anti-IE1A IgG and MS is due to differences in HLA frequencies between MS patients and controls. This can be done by adding the most associated HLA allele for each associated haplotype to the regression models when analyzing association of antibodies with affection status.

The association between anti-viral antibody responses and MS is interesting and can give clues to the disease pathology, but it cannot be used for finding the mechanism behind the observed association. The long term aim of this project would be to investigate the role of HHV-6A and HHV-6B in MS disease in order to find if, and possibly how, these viruses can play a role in triggering or activity of the disease.

4.4.6 Incorporation hypothesis

If the higher anti-IE1A levels observed in pre-MS cases can be a sign of a pathologic role of HHV-6A in MS, what could be the mechanism? One hypothesis is the "incorporation hypothesis" [107]. As described in section 1.1.1.2, enveloped viruses not only contain virus proteins on their surface but also incorporated host cell proteins. This could mean that HHV-6A virions released from oligodendrocytes contain myelin proteins. This could potentially activate both T cell responses and B cell responses (Figure 22). Dendritic cells (DCs) are key players in the activation of T cells; if a DC engulf a virus particle it can potentially present peptides derived from any of the virion proteins including the incorporated host proteins on their MHC class II molecules. The virus might also induce the co-stimulatory signals needed for DC activation of naïve autoimmune T cells. Activation of B cells does not involve DCs but primarily depends on activation by T helper cells. As described in section 1.2, binding of an antigen to the BCR will induce internalization of the BCR-antigen complex and peptides derived from the antigen will be presented on MHC class II molecules where they can be recognized by T cell receptors. Important to remember is that the BCR-specificity does not need to reflect what is presented on the MHC molecule, i.e. a B cell specific for a host cell protein can be activated by a T helper cell because of presentation of virus peptides to the T cell.

According to text books, T and B cells specific against host epitopes should be deleted or developed into regulatory or unresponsive cells during central and peripheral tolerance processes (reviewed in [183, 184]). The incorporation hypothesis, and the other hypotheses of triggering of autoimmunity, relies on the assumption that there are immune cells with specificity against host proteins that have escaped these tolerance mechanisms. It has been implicated that immune cells with self-specificity is present in all individuals but that individuals with autoimmune diseases have more breaking of central and peripheral tolerance [184, 185]. However, even though cells can be specific for self-epitopes, they still need to get activated before they exert their effector function. The incorporation hypothesis also relies on the assumption that virus particles are released from the target cells, and that myelin proteins are incorporated in the virions. This needs to be clarified in the case of HHV-6A in oligodendrocytes.

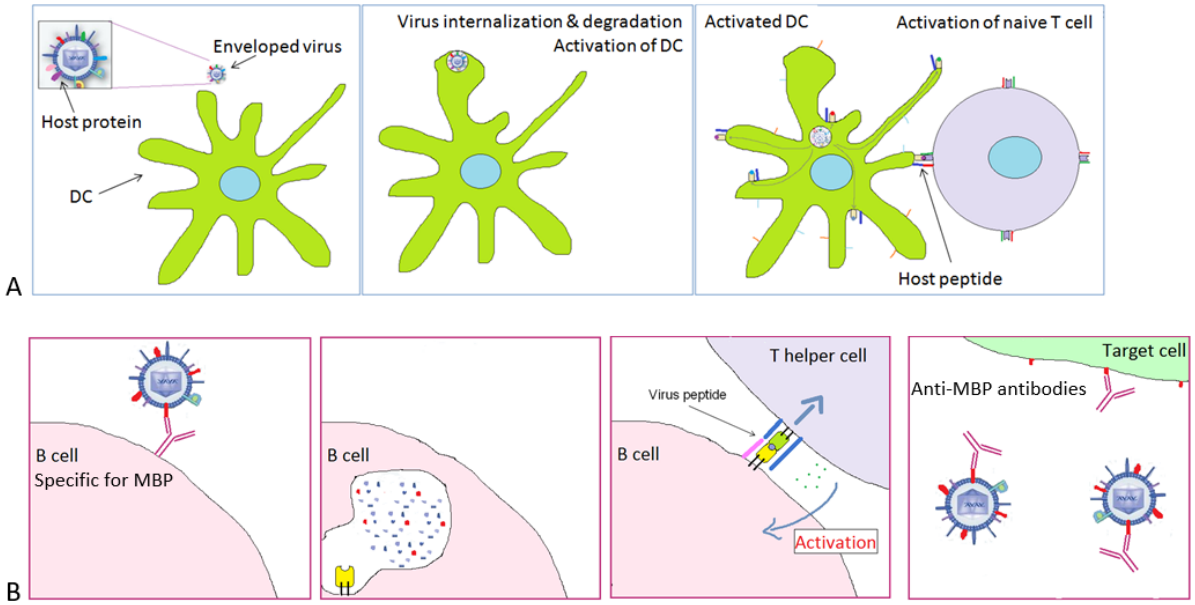


Figure 22: Two hypothetic and simplified ways in which enveloped viruses induce autoimmunity. Activation of autoreactive A) T cells and B) B cells. DC = Dendritic cell. MBP = myelin basic protein, one example of host cell protein.

The incorporation hypothesis explains the cell type specificity in brain and also that that activated immune cells find their way to the target area as there will be signs of inflammation making the immune cell adhere and cross endothelial barriers. In addition, at the target site in the brain, T cells need to be reactivated in order to attack their target and budding of viral particles in the area could trigger antigen presenting cells in the brain to reactivate these effector cells.

5 THESIS SUMMARY

This thesis spans over several angles of HHV-6A and HHV-6B research but has three major focus areas:

Assay validation: The first two studies are focused on improving quality of research. First, in study I we developed and compared different titer determining assays for HHV-6A. This study proved qPCR to be a robust readout for the $TCID_{50}$ assay. In Study II, we searched for a gene with stable expression during HHV-6B infection to be used in gene expression analyses. This study proved PPIA to be stably expressed and therefore suitable to use as reference gene.

Virus-host interaction: Study III focused on how HHV-6B affects the host cell DNA methylation. We could, for the first time, show that HHV-6B induces hypomethylation close to the telomeres and that this process most likely plays a role in virus integration into the host cell genome. In study V, the association between HLA alleles and the IgG response against HHV-6 specific epitopes was investigated. This study revealed that the host HLA genotype plays an important role in shaping the specificity of the elicited anti-HHV-6A/6B antibody response.

Clinical relevance: In study III, we investigated the role of HHV-6B on DNA methylation in epileptic tissue, with the aim of finding a pathogenic role of this virus in epilepsy. Unfortunately, no firm conclusion can be drawn from this investigation due to low power of the analysis. More successfully, we investigated antibodies against HHV-6A and HHV-6B in multiple sclerosis patients and controls in study V. We could, for the first time, show that anti-IE1A and anti-101K IgG responses are increased in MS patients and these responses may indicate a role of these viruses in MS pathology.

To conclude, we have found that HHV-6B affects the host cell epigenetic landscape possibly leading to health consequences. However, proving a link between ubiquitous viruses like HHV-6A and HHV-6B and diseases is hard as several factors are involved. To be infected is not enough to cause diseases like MS and epilepsy, and what influences the virus-host interaction is therefore of interest. Even though these viruses have been associated to different diseases, finding the mechanisms behind the reported associations are desirable and the only way to understand what the associations reflect. Finding pathogenic mechanisms can ultimately lead to better diagnostics and new much more specific treatment options.

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7 REFERENCES

1. Salahuddin, S.Z., et al., *Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders.* Science, 1986. **234**(4776): p. 596-601.
2. Ablashi, D.V., et al., *HBLV (or HHV-6) in human cell lines.* Nature, 1987. **329**(6136): p. 207.
3. Ablashi, D.V., et al., *Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates.* Virology, 1991. **184**(2): p. 545-52.
4. Adams, M.J. and E.B. Carstens, *Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2012).* Arch Virol, 2012. **157**(7): p. 1411-22.
5. Yamanishi, K., et al., *Identification of human herpesvirus-6 as a causal agent for exanthem subitum.* Lancet, 1988. **1**(8594): p. 1065-7.
6. Dewhurst, S., et al., *Human herpesvirus 6 (HHV-6) variant B accounts for the majority of symptomatic primary HHV-6 infections in a population of U.S. infants.* J Clin Microbiol, 1993. **31**(2): p. 416-8.
7. Hall, C.B., et al., *Human herpesvirus-6 infection in children. A prospective study of complications and reactivation.* N Engl J Med, 1994. **331**(7): p. 432-8.
8. Zerr, D.M., et al., *A population-based study of primary human herpesvirus 6 infection.* N Engl J Med, 2005. **352**(8): p. 768-76.
9. Okuno, T., et al., *Seroepidemiology of human herpesvirus 6 infection in normal children and adults.* J Clin Microbiol, 1989. **27**(4): p. 651-3.
10. Hall, C.B., et al., *Characteristics and acquisition of human herpesvirus (HHV) 7 infections in relation to infection with HHV-6.* J Infect Dis, 2006. **193**(8): p. 1063-9.
11. Ward, K.N., et al., *IgG antibodies to human herpesvirus-6 in young children: changes in avidity of antibody correlate with time after infection.* J Med Virol, 1993. **39**(2): p. 131-8.
12. Grinde, B., *Herpesviruses: latency and reactivation - viral strategies and host response.* J Oral Microbiol, 2013. **5**.
13. Hill, J.A. and D.M. Zerr, *Roseoloviruses in transplant recipients: clinical consequences and prospects for treatment and prevention trials.* Curr Opin Virol, 2014. **9**: p. 53-60.
14. Grove, J. and M. Marsh, *The cell biology of receptor-mediated virus entry.* J Cell Biol, 2011. **195**(7): p. 1071-82.
15. Santoro, F., et al., *CD46 is a cellular receptor for human herpesvirus 6.* Cell, 1999. **99**(7): p. 817-27.
16. Ablashi, D., et al., *Classification of HHV-6A and HHV-6B as distinct viruses.* Arch Virol, 2014. **159**(5): p. 863-70.
17. Tang, H., et al., *Detailed study of the interaction between human herpesvirus 6B glycoprotein complex and its cellular receptor, human CD134.* J Virol, 2014. **88**(18): p. 10875-82.
18. Mori, Y., et al., *Human herpesvirus 6 variant A but not variant B induces fusion from without in a variety of human cells through a human herpesvirus 6 entry receptor, CD46.* J Virol, 2002. **76**(13): p. 6750-61.
19. Tang, H., et al., *CD134 is a cellular receptor specific for human herpesvirus-6B entry.* Proc Natl Acad Sci U S A, 2013. **110**(22): p. 9096-9.
20. Watts, T.H., *TNF/TNFR family members in costimulation of T cell responses.* Annu Rev Immunol, 2005. **23**: p. 23-68.
21. Black, J.B., et al., *Growth properties of human herpesvirus-6 strain Z29.* J Virol Methods, 1989. **26**(2): p. 133-45.
22. De Bolle, L., et al., *Quantitative analysis of human herpesvirus 6 cell tropism.* J Med Virol, 2005. **75**(1): p. 76-85.

23. Chan, P.K., et al., *Prevalence and distribution of human herpesvirus 6 variants A and B in adult human brain.* J Med Virol, 2001. **64**(1): p. 42-6.
24. Chan, P.K., et al., *Presence of human herpesviruses 6, 7, and 8 DNA sequences in normal brain tissue.* J Med Virol, 1999. **59**(4): p. 491-5.
25. Cuomo, L., et al., *Human herpesvirus 6 infection in neoplastic and normal brain tissue.* J Med Virol, 2001. **63**(1): p. 45-51.
26. Challoner, P.B., et al., *Plaque-associated expression of human herpesvirus 6 in multiple sclerosis.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7440-4.
27. Dietrich, J., et al., *Infection with an endemic human herpesvirus disrupts critical glial precursor cell properties.* J Neurosci, 2004. **24**(20): p. 4875-83.
28. Ahlqvist, J., et al., *Complete replication cycle and acquisition of tegument in nucleus of human herpesvirus 6A in astrocytes and in T-cells.* J Med Virol, 2006. **78**(12): p. 1542-53.
29. Donati, D., et al., *Variant-specific tropism of human herpesvirus 6 in human astrocytes.* J Virol, 2005. **79**(15): p. 9439-48.
30. Ahlqvist, J., et al., *Differential tropism of human herpesvirus 6 (HHV-6) variants and induction of latency by HHV-6A in oligodendrocytes.* J Neurovirol, 2005. **11**(4): p. 384-94.
31. Pedersen, S.M., et al., *Induction of cell-cell fusion from without by human herpesvirus 6B.* J Virol, 2006. **80**(19): p. 9916-20.
32. Liu, F. and Z.H. Zhou, *Comparative virion structures of human herpesviruses,* in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis,* A. Arvin, et al., Editors. 2007: Cambridge.
33. Guo, H., et al., *Role of tegument proteins in herpesvirus assembly and egress.* Protein Cell, 2010. **1**(11): p. 987-98.
34. Lodish, H.F. and M. Porter, *Specific incorporation of host cell surface proteins into budding vesicular stomatitis virus particles.* Cell, 1980. **19**(1): p. 161-9.
35. Klenk, H.D. and P.W. Choppin, *Lipids of plasma membranes of monkey and hamster kidney cells and of parainfluenza virions grown in these cells.* Virology, 1969. **38**(2): p. 255-68.
36. Stoermer, K.A. and T.E. Morrison, *Complement and viral pathogenesis.* Virology, 2011. **411**(2): p. 362-73.
37. Spiller, O.B., et al., *Neutralization of cytomegalovirus virions: the role of complement.* J Infect Dis, 1997. **176**(2): p. 339-47.
38. Saifuddin, M., et al., *Human immunodeficiency virus type 1 incorporates both glycosyl phosphatidylinositol-anchored CD55 and CD59 and integral membrane CD46 at levels that protect from complement-mediated destruction.* J Gen Virol, 1997. **78 (Pt 8)**: p. 1907-11.
39. Hammarstedt, M., et al., *Purification of infectious human herpesvirus 6A virions and association of host cell proteins.* Virol J, 2007. **4**: p. 101.
40. Isegawa, Y., et al., *Comparison of the complete DNA sequences of human herpesvirus 6 variants A and B.* J Virol, 1999. **73**(10): p. 8053-63.
41. Dominguez, G., et al., *Human herpesvirus 6B genome sequence: coding content and comparison with human herpesvirus 6A.* J Virol, 1999. **73**(10): p. 8040-52.
42. Gompels, U.A. and H.A. Macaulay, *Characterization of human telomeric repeat sequences from human herpesvirus 6 and relationship to replication.* J Gen Virol, 1995. **76 (Pt 2)**: p. 451-8.
43. Thomson, B.J., S. Dewhurst, and D. Gray, *Structure and heterogeneity of the a sequences of human herpesvirus 6 strain variants U1102 and Z29 and identification of human telomeric repeat sequences at the genomic termini.* J Virol, 1994. **68**(5): p. 3007-14.

44. Achour, A., et al., *Length variability of telomeric repeat sequences of human herpesvirus 6 DNA.* J Virol Methods, 2009. **159**(1): p. 127-30.
45. Mirandola, P., et al., *Temporal mapping of transcripts in herpesvirus 6 variants.* J Virol, 1998. **72**(5): p. 3837-44.
46. Oster, B. and P. Hollsberg, *Viral gene expression patterns in human herpesvirus 6B-infected T cells.* J Virol, 2002. **76**(15): p. 7578-86.
47. Gravel, A., J. Gosselin, and L. Flamand, *Human Herpesvirus 6 immediate-early 1 protein is a sumoylated nuclear phosphoprotein colocalizing with promyelocytic leukemia protein-associated nuclear bodies.* J Biol Chem, 2002. **277**(22): p. 19679-87.
48. Stanton, R., G.W. Wilkinson, and J.D. Fox, *Analysis of human herpesvirus-6 IE1 sequence variation in clinical samples.* J Med Virol, 2003. **71**(4): p. 578-84.
49. Martin, M.E., et al., *Identification of a transactivating function mapping to the putative immediate-early locus of human herpesvirus 6.* J Virol, 1991. **65**(10): p. 5381-90.
50. Jaworska, J., A. Gravel, and L. Flamand, *Divergent susceptibilities of human herpesvirus 6 variants to type I interferons.* Proc Natl Acad Sci U S A, 2010. **107**(18): p. 8369-74.
51. Pellett, P.E., et al., *A strongly immunoreactive virion protein of human herpesvirus 6 variant B strain Z29: identification and characterization of the gene and mapping of a variant-specific monoclonal antibody reactive epitope.* Virology, 1993. **195**(2): p. 521-31.
52. Mahmoud, N.F., et al., *Human herpesvirus 6 U11 protein is critical for virus infection.* Virology, 2016. **489**: p. 151-7.
53. Yamamoto, M., et al., *Identification of a nucleocapsid protein as a specific serological marker of human herpesvirus 6 infection.* J Clin Microbiol, 1990. **28**(9): p. 1957-62.
54. Hall, C.B., et al., *Persistence of human herpesvirus 6 according to site and variant: possible greater neurotropism of variant A.* Clin Infect Dis, 1998. **26**(1): p. 132-7.
55. Higashimoto, Y., et al., *Development of a human herpesvirus 6 species-specific immunoblotting assay.* J Clin Microbiol, 2012. **50**(4): p. 1245-51.
56. Leibovitch, E.C., et al., *Coinfection of human herpesviruses 6A (HHV-6A) and HHV-6B as demonstrated by novel digital droplet PCR assay.* PLoS One, 2014. **9**(3): p. e92328.
57. Akhyani, N., et al., *Tissue distribution and variant characterization of human herpesvirus (HHV)-6: increased prevalence of HHV-6A in patients with multiple sclerosis.* J Infect Dis, 2000. **182**(5): p. 1321-5.
58. Cone, R.W., et al., *Human herpesvirus 6 in lung tissue from patients with pneumonitis after bone marrow transplantation.* N Engl J Med, 1993. **329**(3): p. 156-61.
59. Cone, R.W., et al., *Coinfection with human herpesvirus 6 variants A and B in lung tissue.* J Clin Microbiol, 1996. **34**(4): p. 877-81.
60. Derfuss, T., R. Hohlfeld, and E. Meinl, *Intrathecal antibody (IgG) production against human herpesvirus type 6 occurs in about 20% of multiple sclerosis patients and might be linked to a polyspecific B-cell response.* J Neurol, 2005. **252**(8): p. 968-71.
61. Ortega-Madueno, I., et al., *Anti-human herpesvirus 6A/B IgG correlates with relapses and progression in multiple sclerosis.* PLoS One, 2014. **9**(8): p. e104836.
62. Politou, M., et al., *Seroprevalence of HHV-6 and HHV-8 among blood donors in Greece.* Virol J, 2014. **11**: p. 153.
63. Sundstrom, P., et al., *An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study.* Neurology, 2004. **62**(12): p. 2277-82.
64. Virtanen, J.O., et al., *Evidence for human herpesvirus 6 variant A antibodies in multiple sclerosis: diagnostic and therapeutic implications.* J Neurovirol, 2007. **13**(4): p. 347-52.

65. Arbuckle, J.H., et al., *The latent human herpesvirus-6A genome specifically integrates in telomeres of human chromosomes in vivo and in vitro.* Proc Natl Acad Sci U S A, 2010. **107**(12): p. 5563-8.
66. Arbuckle, J.H., et al., *Mapping the telomere integrated genome of human herpesvirus 6A and 6B.* Virology, 2013. **442**(1): p. 3-11.
67. Wallaschek, N., et al., *The Telomeric Repeats of Human Herpesvirus 6A (HHV-6A) Are Required for Efficient Virus Integration.* PLoS Pathog, 2016. **12**(5): p. e1005666.
68. Rotola, A., et al., *U94 of human herpesvirus 6 is expressed in latently infected peripheral blood mononuclear cells and blocks viral gene expression in transformed lymphocytes in culture.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13911-6.
69. Thomson, B.J., S. Efstathiou, and R.W. Honess, *Acquisition of the human adeno-associated virus type-2 rep gene by human herpesvirus type-6.* Nature, 1991. **351**(6321): p. 78-80.
70. Trempe, F., et al., *Characterization of human herpesvirus 6A/B U94 as ATPase, helicase, exonuclease and DNA-binding proteins.* Nucleic Acids Res, 2015. **43**(12): p. 6084-98.
71. Wallaschek, N., et al., *The putative U94 integrase is dispensable for human herpesvirus 6 (HHV-6) chromosomal integration.* J Gen Virol, 2016.
72. Morris, C., et al., *Fine mapping of an apparently targeted latent human herpesvirus type 6 integration site in chromosome band 17p13.3.* J Med Virol, 1999. **58**(1): p. 69-75.
73. Nacheva, E.P., et al., *Human herpesvirus 6 integrates within telomeric regions as evidenced by five different chromosomal sites.* J Med Virol, 2008. **80**(11): p. 1952-8.
74. Torelli, G., et al., *Targeted integration of human herpesvirus 6 in the p arm of chromosome 17 of human peripheral blood mononuclear cells in vivo.* J Med Virol, 1995. **46**(3): p. 178-88.
75. Tweedy, J., et al., *Analyses of germline, chromosomally integrated human herpesvirus 6A and B genomes indicate emergent infection and new inflammatory mediators.* J Gen Virol, 2015. **96**(Pt 2): p. 370-89.
76. Tweedy, J., et al., *Complete Genome Sequence of Germline Chromosomally Integrated Human Herpesvirus 6A and Analyses Integration Sites Define a New Human Endogenous Virus with Potential to Reactivate as an Emerging Infection.* Viruses, 2016. **8**(1).
77. Morissette, G. and L. Flamand, *Herpesviruses and chromosomal integration.* J Virol, 2010. **84**(23): p. 12100-9.
78. Pellett, P.E., et al., *Chromosomally integrated human herpesvirus 6: questions and answers.* Rev Med Virol, 2012. **22**(3): p. 144-55.
79. Strenger, V., et al., *Detection of HHV-6-specific mRNA and antigens in PBMCs of individuals with chromosomally integrated HHV-6 (ciHHV-6).* Clin Microbiol Infect, 2014. **20**(10): p. 1027-32.
80. Seifert, M. and R. Kuppers, *Human memory B cells.* Leukemia, 2016.
81. Janeway C.A., T.P., Walport M., Shlomchik M. , *Immunobiology: the immune system in health and disease*. 2005, Garland Science Publishing. p. 367-408.
82. Nutt, S.L., et al., *The generation of antibody-secreting plasma cells.* Nat Rev Immunol, 2015. **15**(3): p. 160-71.
83. Radbruch, A., et al., *Competence and competition: the challenge of becoming a long-lived plasma cell.* Nat Rev Immunol, 2006. **6**(10): p. 741-50.
84. Vidarsson, G., G. Dekkers, and T. Rispens, *IgG subclasses and allotypes: from structure to effector functions.* Front Immunol, 2014. **5**: p. 520.
85. Marsh, S.G., et al., *Nomenclature for factors of the HLA system, 2010.* Tissue Antigens, 2010. **75**(4): p. 291-455.
86. Compston, A. and A. Coles, *Multiple sclerosis.* Lancet, 2002. **359**(9313): p. 1221-31.

87. Dendrou, C.A., L. Fugger, and M.A. Friese, *Immunopathology of multiple sclerosis.* Nat Rev Immunol, 2015. **15**(9): p. 545-58.
88. International Multiple Sclerosis Genetics, C., et al., *Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis.* Nature, 2011. **476**(7359): p. 214-9.
89. Brynedal, B., et al., *HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis.* PLoS One, 2007. **2**(7): p. e664.
90. Hillert, J. and O. Olerup, *Multiple sclerosis is associated with genes within or close to the HLA-DR-DQ subregion on a normal DR15,DQ6,Dw2 haplotype.* Neurology, 1993. **43**(1): p. 163-8.
91. Fogdell-Hahn, A., et al., *Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease.* Tissue Antigens, 2000. **55**(2): p. 140-8.
92. Hedstrom, A.K., et al., *Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis.* Neurology, 2009. **73**(9): p. 696-701.
93. Ascherio, A., K.L. Munger, and K.C. Simon, *Vitamin D and multiple sclerosis.* Lancet Neurol, 2010. **9**(6): p. 599-612.
94. Kakalacheva, K., C. Munz, and J.D. Lunemann, *Viral triggers of multiple sclerosis.* Biochim Biophys Acta, 2011. **1812**(2): p. 132-40.
95. Bjornevik, K., et al., *Negative interaction between smoking and EBV in the risk of multiple sclerosis: The EnvIMS study.* Mult Scler, 2016.
96. Sundqvist, E., et al., *Epstein-Barr virus and multiple sclerosis: interaction with HLA.* Genes Immun, 2012. **13**(1): p. 14-20.
97. Dooley, M.M., et al., *The increased antibody response to Epstein-Barr virus in multiple sclerosis is restricted to selected virus proteins.* J Neuroimmunol, 2016. **299**: p. 147-151.
98. Kvistad, S., et al., *Antibodies to Epstein-Barr virus and MRI disease activity in multiple sclerosis.* Mult Scler, 2014. **20**(14): p. 1833-40.
99. Alvarez-Lafuente, R., et al., *Relapsing-remitting multiple sclerosis and human herpesvirus 6 active infection.* Arch Neurol, 2004. **61**(10): p. 1523-7.
100. Rotola, A., et al., *Human herpesvirus 6 infects the central nervous system of multiple sclerosis patients in the early stages of the disease.* Mult Scler, 2004. **10**(4): p. 348-54.
101. Soldan, S.S., et al., *Increased lymphoproliferative response to human herpesvirus type 6A variant in multiple sclerosis patients.* Ann Neurol, 2000. **47**(3): p. 306-13.
102. Friedman, J.E., et al., *The association of the human herpesvirus-6 and MS.* Mult Scler, 1999. **5**(5): p. 355-62.
103. Virtanen, J.O., et al., *Intrathecal human herpesvirus 6 antibodies in multiple sclerosis and other demyelinating diseases presenting as oligoclonal bands in cerebrospinal fluid.* J Neuroimmunol, 2011. **237**(1-2): p. 93-7.
104. Alvarez-Lafuente, R., et al., *Clinical parameters and HHV-6 active replication in relapsing-remitting multiple sclerosis patients.* J Clin Virol, 2006. **37 Suppl 1**: p. S24-6.
105. Villoslada, P., et al., *The immune response against herpesvirus is more prominent in the early stages of MS.* Neurology, 2003. **60**(12): p. 1944-8.
106. Chastain, E.M. and S.D. Miller, *Molecular mimicry as an inducing trigger for CNS autoimmune demyelinating disease.* Immunol Rev, 2012. **245**(1): p. 227-38.
107. Naucler, C.S., S. Larsson, and E. Moller, *A novel mechanism for virus-induced autoimmunity in humans.* Immunol Rev, 1996. **152**: p. 175-92.
108. Alping, P., et al., *Rituximab versus fingolimod after natalizumab in multiple sclerosis patients.* Ann Neurol, 2016. **79**(6): p. 950-8.
109. Salzer, J., et al., *Rituximab in multiple sclerosis: A retrospective observational study on safety and efficacy.* Neurology, 2016.

110. Sorensen, P.S. and M. Blinkenberg, *The potential role for ocrelizumab in the treatment of multiple sclerosis: current evidence and future prospects.* Ther Adv Neurol Disord, 2016. **9**(1): p. 44-52.
111. Donati, D., et al., *Detection of human herpesvirus-6 in mesial temporal lobe epilepsy surgical brain resections.* Neurology, 2003. **61**(10): p. 1405-11.
112. Fotheringham, J., et al., *Association of human herpesvirus-6B with mesial temporal lobe epilepsy.* PLoS Med, 2007. **4**(5): p. e180.
113. Karatas, H., et al., *Investigation of HSV-1, HSV-2, CMV, HHV-6 and HHV-8 DNA by real-time PCR in surgical resection materials of epilepsy patients with mesial temporal lobe sclerosis.* J Neurol Sci, 2008. **264**(1-2): p. 151-6.
114. Li, J.M., et al., *Detection of human herpes virus 6B in patients with mesial temporal lobe epilepsy in West China and the possible association with elevated NF-kappaB expression.* Epilepsy Res, 2011. **94**(1-2): p. 1-9.
115. Fotheringham, J., et al., *Human herpesvirus 6 (HHV-6) induces dysregulation of glutamate uptake and transporter expression in astrocytes.* J Neuroimmune Pharmacol, 2008. **3**(2): p. 105-16.
116. Lopez-Bayghen, E. and A. Ortega, *Glial glutamate transporters: New actors in brain signaling.* IUBMB life, 2011. **63**(10): p. 816-23.
117. Cavus, I., et al., *Extracellular metabolites in the cortex and hippocampus of epileptic patients.* Annals of neurology, 2005. **57**(2): p. 226-35.
118. Kobow, K., et al., *Increased reelin promoter methylation is associated with granule cell dispersion in human temporal lobe epilepsy.* Journal of neuropathology and experimental neurology, 2009. **68**(4): p. 356-64.
119. Roopra, A., R. Dingledine, and J. Hsieh, *Epigenetics and epilepsy.* Epilepsia, 2012. **53 Suppl 9**: p. 2-10.
120. Zhu, Q., et al., *Increased Expression of DNA methyltransferase 1 and 3a in Human Temporal Lobe Epilepsy.* Journal of molecular neuroscience : MN, 2011.
121. Sarac, S., et al., *Excitatory amino acid transporters EAAT-1 and EAAT-2 in temporal lobe and hippocampus in intractable temporal lobe epilepsy.* APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 2009. **117**(4): p. 291-301.
122. Jawahar, M.C., et al., *Epigenetic alterations following early postnatal stress: a review on novel aetiological mechanisms of common psychiatric disorders.* Clin Epigenetics, 2015. **7**: p. 122.
123. Ernberg, I., M. Karimi, and T.J. Ekstrom, *Epigenetic mechanisms as targets and companions of viral assaults.* Ann N Y Acad Sci, 2011. **1230**: p. E29-36.
124. Lillycrop, K.A. and G.C. Burdge, *Maternal diet as a modifier of offspring epigenetics.* J Dev Orig Health Dis, 2015. **6**(2): p. 88-95.
125. Gao, X., et al., *DNA methylation changes of whole blood cells in response to active smoking exposure in adults: a systematic review of DNA methylation studies.* Clin Epigenetics, 2015. **7**: p. 113.
126. Ntanasis-Stathopoulos, J., et al., *Epigenetic regulation on gene expression induced by physical exercise.* J Musculoskelet Neuronal Interact, 2013. **13**(2): p. 133-46.
127. El-Osta, A. and A.P. Wolffe, *DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease.* Gene Expr, 2000. **9**(1-2): p. 63-75.
128. Subramaniam, D., et al., *DNA methyltransferases: a novel target for prevention and therapy.* Front Oncol, 2014. **4**: p. 80.
129. Huang, Y. and A. Rao, *Connections between TET proteins and aberrant DNA modification in cancer.* Trends Genet, 2014. **30**(10): p. 464-74.
130. Kohli, R.M. and Y. Zhang, *TET enzymes, TDG and the dynamics of DNA demethylation.* Nature, 2013. **502**(7472): p. 472-9.

131. Hoelzer, K., L.A. Shackelton, and C.R. Parrish, *Presence and role of cytosine methylation in DNA viruses of animals.* Nucleic Acids Res, 2008. **36**(9): p. 2825-37.
132. Sinclair, J., *Chromatin structure regulates human cytomegalovirus gene expression during latency, reactivation and lytic infection.* Biochim Biophys Acta, 2010. **1799**(3-4): p. 286-95.
133. Van Opdenbosch, N., H. Favoreel, and G.R. Van de Walle, *Histone modifications in herpesvirus infections.* Biol Cell, 2012. **104**(3): p. 139-64.
134. Takacs, M., et al., *Epigenetic regulation of latent Epstein-Barr virus promoters.* Biochim Biophys Acta, 2010. **1799**(3-4): p. 228-35.
135. Tao, Q. and K.D. Robertson, *Stealth technology: how Epstein-Barr virus utilizes DNA methylation to cloak itself from immune detection.* Clin Immunol, 2003. **109**(1): p. 53-63.
136. Choi, K.H., et al., *Activation of CMV promoter-controlled glycosyltransferase and beta -galactosidase glycogenes by butyrate, tricostatin A, and 5-aza-2'-deoxycytidine.* Glycoconj J, 2005. **22**(1-2): p. 63-9.
137. Esteki-Zadeh, A., et al., *Human cytomegalovirus infection is sensitive to the host cell DNA methylation state and alters global DNA methylation capacity.* Epigenetics, 2012. **7**(6): p. 585-93.
138. Adhya, D. and A. Basu, *Epigenetic modulation of host: new insights into immune evasion by viruses.* J Biosci, 2010. **35**(4): p. 647-63.
139. Matsusaka, K., et al., *Classification of Epstein-Barr virus-positive gastric cancers by definition of DNA methylation epigenotypes.* Cancer Res, 2011. **71**(23): p. 7187-97.
140. Zhao, J., et al., *Genome-wide identification of Epstein-Barr virus-driven promoter methylation profiles of human genes in gastric cancer cells.* Cancer, 2013. **119**(2): p. 304-12.
141. Martinez, A., et al., *Environment-gene interaction in multiple sclerosis: human herpesvirus 6 and MHC2TA.* Hum Immunol, 2007. **68**(8): p. 685-9.
142. Pearson, T.A. and T.A. Manolio, *How to interpret a genome-wide association study.* JAMA, 2008. **299**(11): p. 1335-44.
143. Reich, D.E., et al., *Linkage disequilibrium in the human genome.* Nature, 2001. **411**(6834): p. 199-204.
144. Blomhoff, A., et al., *Linkage disequilibrium and haplotype blocks in the MHC vary in an HLA haplotype specific manner assessed mainly by DRB1*03 and DRB1*04 haplotypes.* Genes Immun, 2006. **7**(2): p. 130-40.
145. Rubicz, R., et al., *Genome-wide genetic investigation of serological measures of common infections.* Eur J Hum Genet, 2015. **23**(11): p. 1544-8.
146. Hammer, C., et al., *Amino Acid Variation in HLA Class II Proteins Is a Major Determinant of Humoral Response to Common Viruses.* Am J Hum Genet, 2015. **97**(5): p. 738-43.
147. Wentworth, B.B. and L. French, *Plaque assay of cytomegalovirus strains of human origin.* Proc Soc Exp Biol Med, 1970. **135**(2): p. 253-8.
148. Reed, L.J., Muench, H., *A simple method of estimating fifty per cent endpoint.* Am. J. Hyg. , 1938. **27**(3): p. 493-497.
149. Jaworska, J., et al., *Inhibition of transcription of the beta interferon gene by the human herpesvirus 6 immediate-early 1 protein.* J Virol, 2007. **81**(11): p. 5737-48.
150. Alvarez-Lafuente, R., et al., *Active human herpesvirus 6 infection in patients with multiple sclerosis.* Arch Neurol, 2002. **59**(6): p. 929-33.
151. Nitsche, A., et al., *Human herpesvirus 6A DNA Is detected frequently in plasma but rarely in peripheral blood leukocytes of patients after bone marrow transplantation.* J Infect Dis, 2001. **183**(1): p. 130-3.
152. Esposito, L., et al., *Large-scale analysis of viral nucleic acid spectrum in temporal lobe epilepsy biopsies.* Epilepsia, 2015. **56**(2): p. 234-43.

153. Du, P., W.A. Kibbe, and S.M. Lin, *lumi: a pipeline for processing Illumina microarray.* Bioinformatics, 2008. **24**(13): p. 1547-8.
154. Radonic, A., et al., *Reference gene selection for quantitative real-time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus and Cytomegalovirus infections.* Virol J, 2005. **2**: p. 7.
155. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
156. Pfaffl, M.W., et al., *Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations.* Biotechnol Lett, 2004. **26**(6): p. 509-15.
157. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. RESEARCH0034.
158. Andersen, C.L., J.L. Jensen, and T.F. Orntoft, *Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets.* Cancer Res, 2004. **64**(15): p. 5245-50.
159. Hillert, J. and L. Stawiarz, *The Swedish MS registry - clinical support tool and scientific resource.* Acta Neurol Scand, 2015. **132**(199): p. 11-9.
160. Hedstrom, A.K., et al., *Smoking and multiple sclerosis susceptibility.* Eur J Epidemiol, 2013. **28**(11): p. 867-74.
161. Holmen, C., et al., *A Swedish national post-marketing surveillance study of natalizumab treatment in multiple sclerosis.* Mult Scler, 2011. **17**(6): p. 708-19.
162. Khademi, M., et al., *Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course.* Mult Scler, 2011. **17**(3): p. 335-43.
163. Waterboer, T., et al., *Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins.* Clin Chem, 2005. **51**(10): p. 1845-53.
164. Price, A.L., et al., *Principal components analysis corrects for stratification in genome-wide association studies.* Nat Genet, 2006. **38**(8): p. 904-9.
165. Chang, C.C., et al., *Second-generation PLINK: rising to the challenge of larger and richer datasets.* Gigascience, 2015. **4**: p. 7.
166. Dilthey, A.T., et al., *HLA*IMP--an integrated framework for imputing classical HLA alleles from SNP genotypes.* Bioinformatics, 2011. **27**(7): p. 968-72.
167. Mori, Y., et al., *Expression of human herpesvirus 6B rep within infected cells and binding of its gene product to the TATA-binding protein in vitro and in vivo.* J Virol, 2000. **74**(13): p. 6096-104.
168. Schleimann, M.H., et al., *Direct Repeat 6 from human herpesvirus-6B encodes a nuclear protein that forms a complex with the viral DNA processivity factor p41.* PLoS One, 2009. **4**(10): p. e7457.
169. Schleimann, M.H., et al., *The DR6 protein from human herpesvirus-6B induces p53-independent cell cycle arrest in G2/M.* Virology, 2014. **452-453**: p. 254-63.
170. Ablashi, D.V., et al., *Human Herpesvirus-6 (HHV-6) infection in multiple sclerosis: a preliminary report.* Mult Scler, 1998. **4**(6): p. 490-6.
171. Agulnick, A.D., et al., *Identification of a DNA-binding protein of human herpesvirus 6, a putative DNA polymerase stimulatory factor.* J Gen Virol, 1993. **74 (Pt 6)**: p. 1003-9.
172. Santiago, O., et al., *Relation between Epstein-Barr virus and multiple sclerosis: analytic study of scientific production.* Eur J Clin Microbiol Infect Dis, 2010. **29**(7): p. 857-66.

173. Dondelinger, Y., et al., *An evolutionary perspective on the necroptotic pathway.* Trends Cell Biol, 2016. **26**(10): p. 721-32.
174. Kunzelmann, K., *Ion channels in regulated cell death.* Cell Mol Life Sci, 2016. **73**(11-12): p. 2387-403.
175. Ofengeim, D., et al., *Activation of necroptosis in multiple sclerosis.* Cell Rep, 2015. **10**(11): p. 1836-49.
176. Brandle, S.M., et al., *Distinct oligoclonal band antibodies in multiple sclerosis recognize ubiquitous self-proteins.* Proc Natl Acad Sci U S A, 2016. **113**(28): p. 7864-9.
177. Kong, H., et al., *Human herpesvirus type 6 indirectly enhances oligodendrocyte cell death.* J Neurovirol, 2003. **9**(5): p. 539-50.
178. Li, Z.K., et al., *The effect of HLA on immunological response to hepatitis B vaccine in healthy people: a meta-analysis.* Vaccine, 2013. **31**(40): p. 4355-61.
179. Martin, L.K., et al., *Specific CD8(+) T cells recognize human herpesvirus 6B.* Eur J Immunol, 2012. **42**(11): p. 2901-12.
180. Masuda, A., et al., *Cis binding between inhibitory receptors and MHC class I can regulate mast cell activation.* J Exp Med, 2007. **204**(4): p. 907-20.
181. Shiroishi, M., et al., *Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G.* Proc Natl Acad Sci U S A, 2003. **100**(15): p. 8856-61.
182. Bashirova, A.A., et al., *LILRB2 interaction with HLA class I correlates with control of HIV-1 infection.* PLoS Genet, 2014. **10**(3): p. e1004196.
183. Abbas A., L.A.H., Pillai S., *Cellular and Molecular Immunology*. 2012, Saunders, an imprint of Elsivier Inc.
184. Gonsette, R.E., *Self-tolerance in multiple sclerosis.* Acta Neurol Belg, 2012. **112**(2): p. 133-40.
185. Samuels, J., et al., *Human B cell tolerance and its failure in rheumatoid arthritis.* Ann N Y Acad Sci, 2005. **1062**: p. 116-26.